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Characterisation of β-lactam/Vaborbactam activity in *Klebsiella pneumoniae*

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ABSTRACT

Carbapenem resistant *Klebsiella pneumoniae* is one of the most clinically relevant multidrug resistant pathogens, exacerbated by an ability to cause nosocomial infections. While treatment choices are limited, the introduction of two β-lactam/β-lactamase inhibitor combinations: meropenem/vaborbactam and ceftazidime/avibactam has revolutionised the management of cephalosporin and carbapenem resistant *K. pneumoniae* infections. With an increasing clinical dependence on these combination therapies, there is a need to study resistance mechanisms to allow prediction of what might be seen in the clinic and perhaps suggest ways to reverse resistance when it occurs. In particular, this project aims to better understand β-lactam/vaborbactam efficacy against laboratory grown bacteria and the mechanisms of resistance that emerge by generating mutants of *K. pneumoniae* clinical isolates with reduced susceptibility to β-lactams in combination with vaborbactam. We showed that vaborbactam is a potent inhibitor of class A and C β-lactamases *in vivo* but shows particular potency against bacteria carrying the class A, KPC carbapenemase, when used in combination with meropenem. Upon analysis of meropenem/vaborbactam resistant mutants, the outer membrane porin OmpK36 was frequently lost, as has been previously reported. We also identified a novel meropenem/vaborbactam resistance mechanism, which involves down-regulation of OmpK36 and the Maltoporin LamB2. An attempt was made to find a universal β-lactam/vaborbactam combination that would be clinically active against meropenem/vaborbactam resistant *K. pneumoniae* producing KPC, CTX-M or the OXA-48 like carbapenemase OXA-232. While imipenem/vaborbactam and ceftazidime/vaborbactam were active against meropenem/vaborbactam mutants of CTX-M and OXA-232 producers, an alternative combination could not be found that was active against a meropenem/vaborbactam resistant mutant derived from a KPC producer. Furthermore, ceftazidime/vaborbactam resistance was identified in CTX-M producers as being caused by over-production of the CTX-M enzyme. Imipenem/vaborbactam resistance was not identified in this background. Finally, demonstrated for the first time that combining avibactam/ceftazidime with meropenem/vaborbactam overcomes meropenem/vaborbactam resistance in all mutants generated. Therefore, we conclude that combining these β-lactam/β-lactamase inhibitor combinations in the same patient shows great potential in the treatment *K. pneumoniae* isolates resistant to one of these combinations

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DEDICATION

This Master's thesis and the whole of my degree is dedicated to my father Colin Frederick Douglas (1957-2013) and my grandfather Frederick Douglas (1931-2018). Two great men loved and adored by everyone that knew them, taken far too soon. They will continue to be sorely missed by my grandmother Barbara, my mother Susan and my siblings Hannah and William. I hope I have made you both proud and that I continue to do so with the start of my PhD. I will continue to try and emulate and pass down your values and our proud family traditions. You have both taught me so much and been such amazing role models. I hope that I can become even a shadow of the men that you were. I miss you more every day.

'Jamais arriere'

AUTHORS DECLARATION

I declare that the work in this thesis was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in text, the work is the candidates own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author. In addition, the e submission and hard copy are identical.

SIGNED: EDWARD JAMES ALEXANDER DOUGLAS DATE 20/09/2018

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ABBREVIATIONS

ABC – ATP binding cassette

ABR – Antibacterial resistance

AvyCaz – Avibactam/Ceftazidime

CLSI – Clinical Laboratory Standards Institute

CRE – Carbapenem resistant Enterobacteriaceae

DHP-1 – Human renal dehydropeptidase 1

DMSO – Dimethyl-sulfoxide

EDTA – Ethylenediaminetetra-acetic acid

ESBL – Extended spectrum β-lactamase

ESKAPE – *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter spp*

GlcNAc – *N*-acetylglucosamine

HMM – High molecular mass

KPC – *Klebsiella pneumoniae* carbapenemase

LB – Luria Bertani

LMM – Low molecular mass

LPS – Lipopolysaccharide

MATE – Multi-drug and toxic efflux

MBL – Metallo-β-lactamase

MDR – Multi-drug resistance

MFS – Major facilitator superfamily

MH – Mueller Hinton

MIC – Minimal inhibitory concentration

MRSA – Methicillin resistant *Staphylococcus aureus*

MurNAc – *N*-acetylmuramic acid

NC – No change

NMR – Nuclear magnetic resonance

OD – Optical density

Omp – Outer membrane protein

ORF – Open reading frame

OXA – Oxacillinase

PBP – Penicillin binding protein

PBS – Phosphate buffer saline

PCR – Polymerase chain reaction

RND – Resistance-nodulation-division

Rpm – revolutions per minute

SBL – Serine β-lactamase

SCC*mec* – Staphylococcal cassette chromosome *mec*

SDS-PAGE – Sodium dodecyl sulphate Polyacrylamide gel electrophoresis

SMR – Small multi-drug resistance

SOC – Super optimal broth

TAE – Tris base, acetic acid, EDTA

TEMED - Tetramethylethylenediamine

UDP – Uridine dihydro diphosphate

UTI – Urinary tract infection

Vabomere – Vaborbactam/Meropenem

VRE – Vancomycin resistant enterococci

WHO – World Health Organisation

“Without urgent, coordinated action by many stakeholders, the world is headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill”

Dr Keiji Fukunda, 2014: Assistant director-general at the WHO

Chapter 1: Introduction

1.1. The History of Antibiotics

Antibiotics, more specifically antibacterials, refer to substances capable of killing bacteria (bactericidal) or inhibiting their growth (bacteriostatic). The term is used generally to encompass both natural as well as synthetic products. The first antibiotic made available for clinical use was the anti-pseudomonal drug; pyocyaneoprotein, later referred to as pyocynase. It was successfully introduced by Ivan Honl (1866-1936) and Jaroslav Bukovský in 1911 to combat infections of the pharynx and larynx and was used continuously until the end of the first world war , where it was discontinued due to concerns over toxicity (1).

Arguably, the most important antibiotic discovery was made in 1928 by Sir Alexander Fleming. Fleming noticed that *Staphylococcus aureus* contaminated with the mould *Penicillium notatum* showed an altered morphology which was due to bacterial cell lysis. He hypothesised that there must be some sort of lytic compound being produced by the *Penicillium* mould, which he subsequently named penicillin (2). However, Fleming was not responsible for the large-scale synthesis, purification and clinical testing of penicillin that confirmed its therapeutic potential as a bactericidal drug and made possible its use in the clinic (3, 4). The credit for this feat is often given to Howard Florey (1898-1968) and Ernst Chain (1906-1979), but, in reality, this would not have been possible if not for Norman Heatley (1911-2004), who perfected the purification of penicillin, allowing sufficient quantities to be made and further tested (5). Penicillin was introduced in the clinic in 1941, where it was used to treat Albert Alexander for a scratch on the inside of his mouth (6). This heralded the first use of possibly the most successful class of antibiotics to date; the β-lactams. Perhaps more importantly, Fleming shone the light onto the phenomenon of antibiosis. This term describes the ability of microorganisms to kill other microorganisms by the production and subsequent secretion of chemicals. The discovery of penicillin was the first observation of this famous phenomenon, that later lead to the discovery of several other antibiotics during the 19th century (7).

The first use of the term ‘antibiotic’ was by Selman Waksman (1888-1973), who was later credited as being the ‘Father of Antibiotics’. The first antibiotic he discovered was Actinomycin which was isolated from *Actinomyces antibioticus* (8, 9). Waksman later worked on the discovery and isolation of the first member of the aminoglycoside antibiotics: streptomycin produced by the common environmental bacteria *Streptomyces griseus.* In total, Waksman identified over 20 new natural antibacterial substances using a screening technique of looking for inhibitory zones of growth around colonies of soil dwelling bacteria (8, 10).

1.2. Current Bacterial cell targets

Importantly, antibiotics must exert their effect with minimum cross reactivity on human cells. This makes it more difficult to find antibiotics that act on bacterial specific pathways. Currently, all antibiotics exploit one of five bacterial targets (11). What is common for all targets is their vital function for bacterial cell growth as interference with the function of any of these targets either results in cell lysis or a negative impact on cellular growth. These are:

1. Bacterial cell wall biosynthesis. Antibiotics that inhibit the bacterial this pathway include the β-lactams (e.g. penicillin see section 1.3.) and glycopeptides (e.g. vancomycin) which inhibit penicillin binding proteins (PBPs) and peptidoglycan monomers respectively (12, 13). Both result in loss of cell shape an osmotic instability leading to bacterial cell lysis.

2. Protein synthesis. Bacterial protein synthesis is sufficiently different to human protein synthesis to allow it to be an effective antibiotic target. Specifically, it is the interaction with the ribosomal subunits that leads to inhibition of protein synthesis. Macrolides (e.g. azithromycin), linezolid and chloramphenicol inhibit the larger 50s subunit. Whereas the smaller 30S ribosomal subunit is the target of aminoglycosides (e.g. streptomycin) and tetracyclines (e.g. minocycline) (11, 14).

3. Nucleic acid synthesis. DNA synthesis is an essential bacterial pathway that can be inhibited by the synthetic quinolones (e.g. ciprofloxacin) which act on DNA topoisomerase II (DNA gyrase) in Gram negative bacteria and a gyrase homologue; DNA topoisomerase IV in Gram positive bacteria. These enzymes are responsible for regulating the torsional stress associated with DNA supercoiling (11, 15). Inhibition of these enzymes results in the sheering of DNA leading to cell death. Contrastingly, rifampicin prevents DNA dependant RNA synthesis by inhibiting DNA dependant RNA polymerase. This stops bacterial transcription from being able to take place (16).

4. Folate synthesis. The folate synthesis pathway is required for nucleotide synthesis and can be inhibited by antibiotics such as trimethoprim and sulphonamides. Trimethoprim inhibits dihydrofolate reductase, preventing the conversion of dihydrofolate to tetrahydrofolate (17). Whereas sulphonamides prevent the conversion of para-aminobenzoic acid into folic acid, through competition for the dihydropteroate synthetase enzyme (18).

5. Cell membrane structure. Finally, polymyxins (e.g. colistin) and daptomycin target the bacterial cell membrane. Polymyxins bind to LPS with a higher affinity than Mg^{2+} and Ca^{2+} ions, which are required for LPS stability. This results in an altered cell membrane permeability leading to cell death (19). Whereas daptomycin binds to LPS, inserting its lipophilic tail which causes membrane depolarisation and a rapid efflux of potassium ions resulting in cell death (20) (21).

1.3. Antibiotic Resistance

Antibiotic or antibacterial resistance (ABR) is a term that describes a phenotype whereby bacteria show a lack of sensitivity to an antibiotic of which they previously were sensitive. Antibiotics are invaluable as a means to cure patients suffering from bacterial infections. Therefore, in the clinic ABR refers to whether or not a specific antibiotic treatment will be able to succeed or indeed fail. ABR can occur due to the acquisition of resistance mechanisms or the presence of existing intrinsic mechanisms that are able to promote survival in the presence of the antibiotic. Resistance mechanisms fall into four main categories: Target site modification (including enzymatic modifications), acquisition of a non-susceptible target, altering outer membrane permeability and enzymatic degradation of the antibiotic (see section 1.4.) (22). It is important to understand that antimicrobials, more specifically antibacterial, are by and large semi-synthetic and show similarity to natural products. Therefore, the evolution of ABR is understood to have occurred naturally over millions of years and is not by any means a new phenomenon. It is thought that ABR mechanisms could have originated in antibiotic producing organisms (such as *Streptomyces griseus*) as a means of protecting themselves against the antibiotics they produce. However, it is clear that the increased utilisation of antibiotics worldwide is accelerating this process and providing a significant selection pressure, leading to the selection of ABR in many bacterial species (23). This is no more important than in the case of hospital acquired infections (infections that are not present at the time of admission and not occurring 48 hours after admittance (24)). Due to the fact that antibiotics select for resistant bacteria, it therefore follows that environments with higher antibiotic levels would have more resistant bacteria (25). Consequently, hospitals tend to have a high incidence of antibiotic resistant bacterial isolates. This is highlighted by the number of infections caused by bacteria resistant to one or more drugs, in the US annually: estimated to be approximately 2 million (26).

ABR has been described as one of the direst threats currently facing the public health sector (27, 28). ABR frequently causes treatment failure, which is often fatal, especially in immunocompromised individuals (29). Indeed, it is estimated that ABR contributes to the deaths of roughly 50,000 people across Europe and the United States and 700,000 people globally (28). However, the full extent of the threat that ABR poses globally is surely an underestimation due to the lack of information and surveillance regarding this current issue. The threat that ABR has on public health is by no means the only issue, the financial burden surrounding increased ABR is estimated to cost the NHS £1.5 billion and $41 billion in the US annually (30).

We have seen the problems of increased mortality, morbidity as well as the increased financial issues surrounding ABR. However, worryingly, this is exacerbated by the lack of discovery of new antimicrobial classes. Figure 1 shows that the last antimicrobial class discovered, which has been used in clinical practice, was the Lipopeptides in 1987, almost 30 years ago, highlighting the need, not only to discover new antibiotics but to also protect our current antibiotics (31, 32). The reason for the reduced number of antibiotics reaching clinical trial is largely due to pharmaceutical companies having little economic incentive to develop drugs that combat infectious disease. Not only are antibiotics prescribed for short treatments, but they also end up being used less and less over time due to the inevitable intervention of antibiotic resistance. Therefore, there is little incentive for pharmaceutical companies to develop antibiotics as the return on investment is likely to be minimal (33).

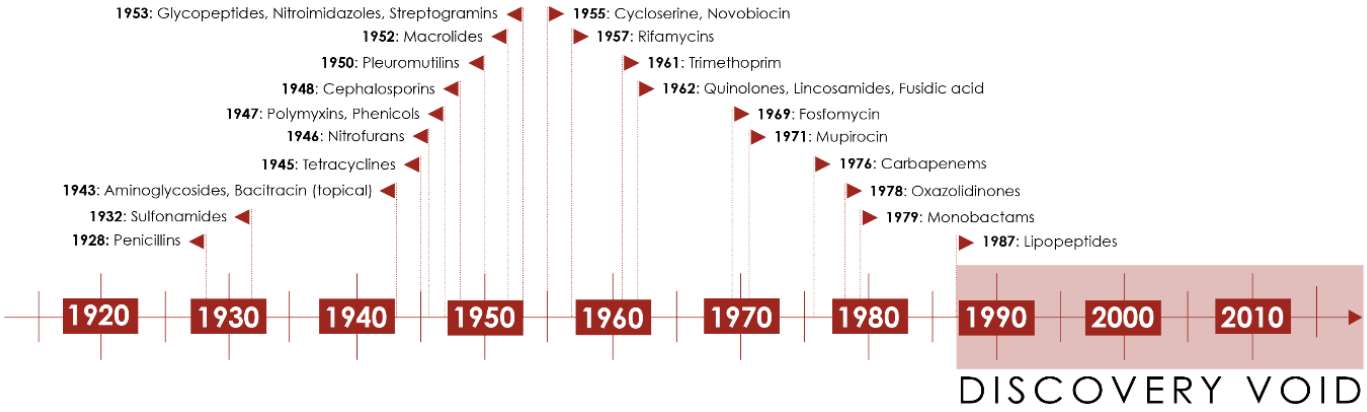

Figure 1. A timeline presenting the 'Golden era' of antibiotic discovery. The first antibiotic discovered was penicillin in 1928, isolated from the mould *Penicillium spp*. Many other naturally derived antibiotics soon followed including: aminoglycosides, glycopeptides, pleuromutilin's etc. Around 20 classes of antibiotics where discovered between 1928 and 1987 leading to this period being referred to as the apt 'Golden era' of antibiotic detection and synthesis. From 1987 onwards, no new antibiotics reached the clinic meaning we are currently in a discovery void and have been for over 30 years. Figure taken from (34).

Additionally, the situation surrounding ABR is not helped by the important roles that antibiotics provide in the marine and agricultural industries. Antibiotics are frequently added to animal feed or water to act as a growth promoter but also to provide a mass prophylaxis treatment to prevent the outbreak of disease (35, 36). It is known that this is having a significant impact on the prevalence of ABR. A great example of this is the case of vancomycin resistance. Vancomycin is a glycopeptide antibiotic produced by the environmental soil bacterium; *Streptomyces oreintalis.* Therefore, vancomycin resistance is common in environmental bacterial species, the most clinically significant of which is *Enterococcus faecium.* The prevalence of vancomycin resistant enterococci (VRE), specially *E. faecium,* in farm animals was found to increase significantly due to the use of a glycopeptide analogue avoparcin as a prophylaxis treatment and growth promoter. Therefore, using avoparcin provided a strong selection pressure for glycopeptide resistant bacteria. Consequently, the use of avoparcin ceased and the prevalence of VRE in farm animals decreased (37).

In conclusion, the threat of ABR is not one that has a simple solution. It is a multifaceted problem consisting of an increase in occurrence of antibiotic resistant bacterial pathogens, exacerbated by the lack of discovery of new antibiotics combined with the increase in utilisation of antibiotics in the public health and agricultural sectors. If ABR is not combatted urgently and universally it is estimated that deaths could rise to as high as 10 million per year by 2050, exceeding the deaths attributed to cancer, costing the healthcare system an additional $100 trillion (28). What is more, since the discovery of penicillin, we have been unable to pinpoint a single antibiotic class which can bypass the development of resistance (25, 38).

1.4. β-lactams

1.4.1. Overview

The antibiotic class of β-lactams includes the 'original' antibiotic penicillin discovered by Sir Alexander Fleming as well as penicillin derivatives and cephalosporins, carbapenems and monobactams. Collectively they make up the most commonly prescribed group of antibiotics and have been estimated to account for up to 53% of total antibiotic sales and consumption globally (figure 2). The reason for their popularity is largely down to their specificity combined with impressive potency as a bactericidal antibiotic. It is for this reason that β-lactams have become vital for the treatment of infections caused by the Gram-negative human pathogens *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa.* It was the elucidation of the structure of penicillin in 1945 by Dorothy Hodgkin that allowed the mechanism of action to be defined. The structure of β-lactams can differ to varying degrees, however all of contain a nitrogen based β-lactam ring. It is their varying ring structures and side chains that lead to the increased activity/potency seen with the newer members of the β-lactams: cephalosporins and carbapenems (figure 3).

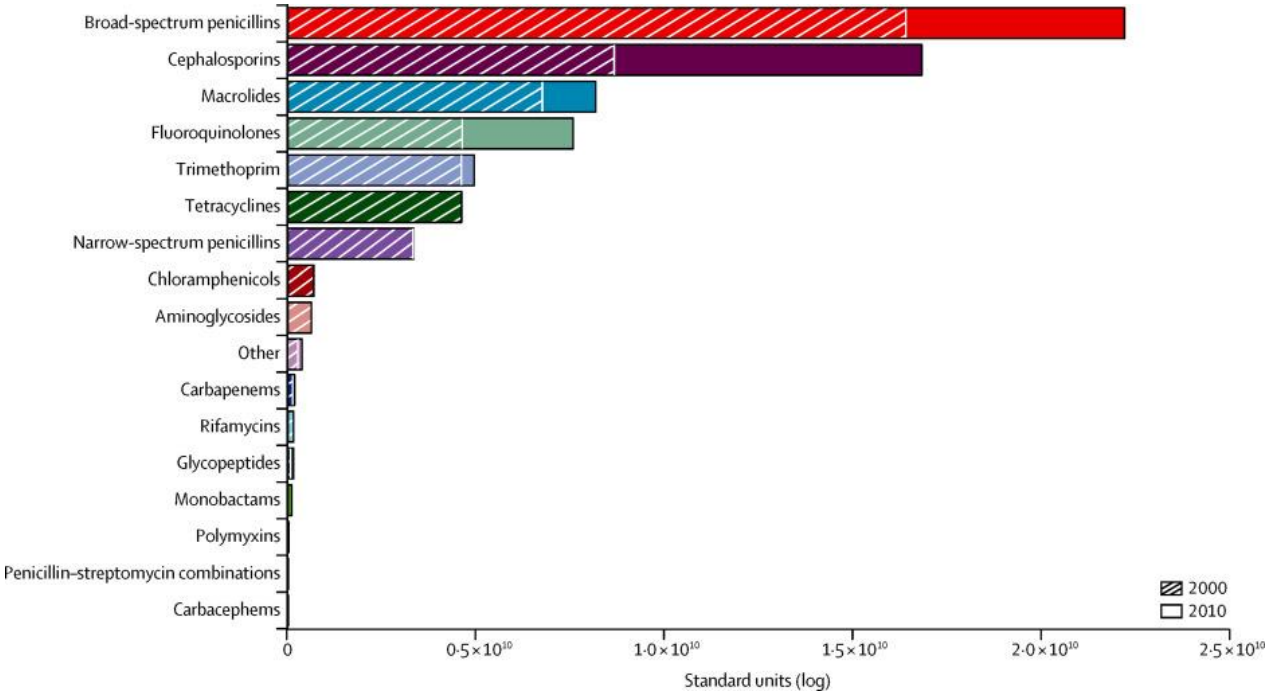

Figure 2. Global antibiotic consumption between 2000 and 2010. Both broad-spectrum penicillins, narrow-spectrum penicillins, cephalosporins, carbapenems and monobactams belong to the β-lactam class of antibiotic. This graph shows the how important β-lactams are and how frequently they are prescribed. The X axis shows consumption as the log of daily dosage units. Figure taken from (23).

1.4.2. β-lactam subgroups

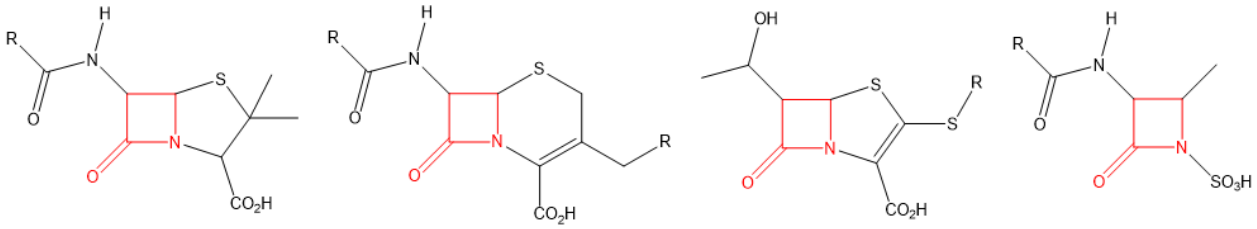

Figure 3. The chemical structure of the four main sub-groups belonging to the antibiotic class of β-lactams. The main ring structures are shown on the right of each antibiotic, with the sidechains denoted by the letter 'R'. We can see that each group within the β-lactam class contain a 4 membered, nitrogen containing β-Lactam ring, highlighted in red. Penicillin contains a thiazolidine ring, cephalosporins contain a dihydrothiazinic ring. We see that carbapenems contain a very similar overall structure to penicillin's, however the Sulphur group in the thiazolidine ring is replaced with a Carbon atom. Monobactams do not contain a side ring (39, 40). Figure adapted from (40). Figure was made using ChemDraw professional 16.0.

1.4.2.1. Penicillins

Penicillin G otherwise called benzylpenicillin (1st generation penicillin) was the original, natural β-lactam initially isolated from the mould *Penicillium notatum* (2). It was the first β-lactam clinically used. It showed a range of activity that only included Gram-positive bacteria, specifically, infections caused by *Streptococcus*, *Staphylococcus* and *Enterococcus* species (41). However, penicillin G showed limited activity against β-lactamase producing Gram-positive cocci, such as *blaZ* (penicillinase) producing *S. aureus* (42). This is because penicillin G showed vulnerability to acid hydrolysis. To combat the action of these enzymes, larger, semi-synthetic penicillins such as methicillin (2nd generation penicillin) were synthesised. Methicillin was able to evade binding of these enzymes, whilst still retaining bactericidal activity (43). However, methicillin still only showed a narrowed spectrum, specific to Gram-positive bacteria. The addition of a hydrophilic group to the α-carbon produced the first member of the aminopenicillins; ampicillin (3rd generation penicillin). ampicillin was the

first penicillin that showed an extended activity and could target both Gram-positive and Gram-negative bacteria (44, 45).

Once the structure of penicillin was solved by Dorothy Hodgkin, it was found that there were several constituents that were central to penicillin's function as a bactericidal antibiotic. These include: the β-lactam ring which is vital as it shows steric similarity to D-Ala-D-Ala (see section 1.4.3.) allowing the acylation of PBPs (46). The carboxyl group at the C3 carbon which facilitates irreversible binding to the PBP active site. The carbonyl group is highly susceptible to nucleophiles as it is prone to losing its' hydrogen and subsequently becoming negatively charged, allowing it to interact with the NH_3+ on the lysine residue present in the PBP active site. The sulphur group is thought to promote the electrophilic potential of the carbonyl group, further facilitating binding to the PBP active site (39, 47, 48). Modifications of the R group present at the C6 carbon give rise to penicillin's with different potency's but also different acid-hydrolysis sensitivity (47, 49).

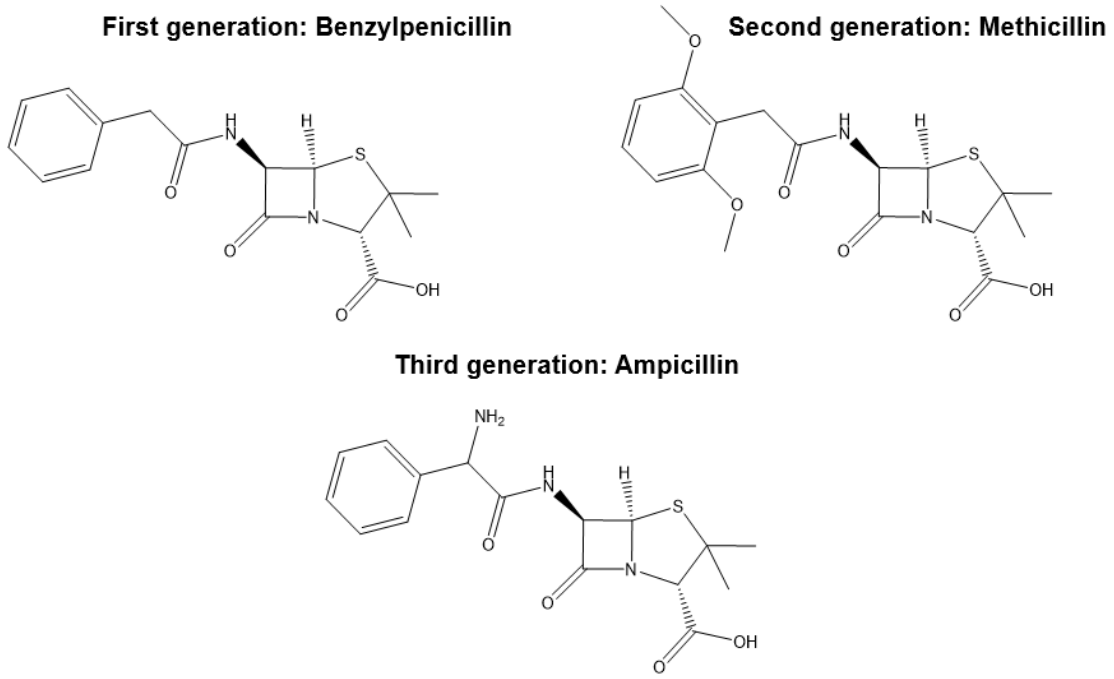

Figure 4. Chemical structures of first, second and third generation penicillins. Figure was made using ChemDraw professional 16.0. All penicillins contain the invariable β-lactam ring fused to a 5-membered thiazolidine ring.

1.4.2.2. Cephalosporins

Even with the synthesis of the aminopenicillins, the activity on Gram-negative pathogens remained limited, largely due to the existence of intrinsic resistance mechanisms (see section 1.5.) and the presence of β-lactamases. This combined with the mobilisation and subsequent dissemination of the TEM-1 and SHV-1 β-lactamases (see section 1.5.) provided a significant blow in the antibiotic 'arms race'. The first cephalosporin, cephalosporin C, was discovered in 1948 from the common saprophytic mold; *Cephalosporium acremonium* (50, 51). Cephalosporins tend to have a much greater spectrum of activity with regards to Gram-negative pathogens. However, this comes at a price, as the Gram-negative activity increases with the later generations of cephalosporins, the Gram-positive activity becomes compromised. While penicillins contain a 5-membered thiazolidine ring, cephalosporins differ in their structure as they contain a 6-membered dihydrothiazinic ring instead (52). Several generations of cephalosporins have been made and grouped based on their *in vitro* activity and chemical structure. Newer generations of cephalosporins show greater activity against Gram-negative bacteria. Cephalosporins remain, to this day, some of the most frequently prescribed antibiotics in the U.S. (53, 54). Historically, cephalosporins have not been prescribed to target infections caused by Gram-positive bacteria (55). An overview of the different generations cephalosporins and their activity is provided below.

1st Generation Cephalosporins. First-generation cephalosporins, such as cephalothin, tend to have a reduced or comparable potency to that of penicillins. However, advantages of cephalosporins include the insensitivity to penicillinases, acid-hydrolysis stability as well as the ability to target Gram-negative bacteria. The first generation cephalosporins show the greatest activity towards Gram-positive bacteria of all the cephalosporins (53).

2nd Generation Cephalosporins: Second-generation drugs show an increased activity against Gram-negative bacteria; however this is at the cost of a reduced activity against Gram-positive bacteria. Second generation cephalosporins include the cephamycins (e.g. cefoxitin) and oxyimino cephalosporins (e.g. cefuroxime). Cephamycins were initially isolated from several members of the *Streptomyces spp.* including *Streptomyces lactamdurans.* Cephamycins differ in their structure from first-generation cephalosporins, these are: a methoxyl group as well as a D-α-aminoadipic acid added to the C7 carbon position. Additionally, cephamycins also show a variety of side chains present at the C3 carbon position. It is thought that these modifications extend the activity against β-lactamase producing Gram-negative bacteria. Activity against members of the *Proteus* genus is particularly high (56-58). Oxyimino cephalosporins contain a methoxyimino group at the α

carbon position of the acylamino chain. They are approved for use against many Gram-positive bacteria such as: *S. aureus* and *Streptococcus pneumoniae.* They are also approved for use against Gram-negative bacteria such as *Escherichia coli* and specifically, *Haemophilus influenzae* (53). Usefully, cefuroxime can be used to treat certain types of meningitis (e.g. caused by *Neisseria sp.*), as it is the only second-generation drug capable of passing the normally restrictive blood-brain barrier (59).

3rd and 4th Generation Cephalosporins: Third (e.g. ceftazidime) and fourth (e.g. cefepime) - generation cephalosporins are generally grouped together as they are both described as showing an extended-spectrum of activity with the only significant difference being their respective Gram-positive activity. Certain members of the third and fourth-generation cephalosporins have impaired Gram-positive activity compared to first and second-generation cephalosporins but even greater Gram-negative activity (53). Similarly to cefuroxime, third-generation cephalosporins can cross the blood-brain barrier meaning they can be used to treat meningitis, caused by a wide variety of organisms (60). Third-generation cephalosporins are usually reserved in the clinic as the first-choice antibiotic for the treatment of gonorrhoea infections (61). Cefepime shows an improved activity against Gram-negative bacterial infections, including those caused by: *Serratia marcescens, Citrobacter freundii* and *Enterobacter cloacae* (62). Importantly, fourth-generations are capable of being used in combination with other antibiotic classes, mainly aminoglycosides and fluoroquinolones for the treatment of persistent *P. aeruginosa* in cystic fibrosis patients (63-65).

5th Generation Cephalosporins: More recently, two fifth-generation cephalosporins have been made: ceftobiprole in 2002 and ceftaroline in 2005. However, their labelling as fifth-generation cephalosporins remains a disputed term. Ceftobiprole was initially developed as an anti-MRSA drug, whereas ceftaroline shows potent anti-pseudomonal activity (53, 66).

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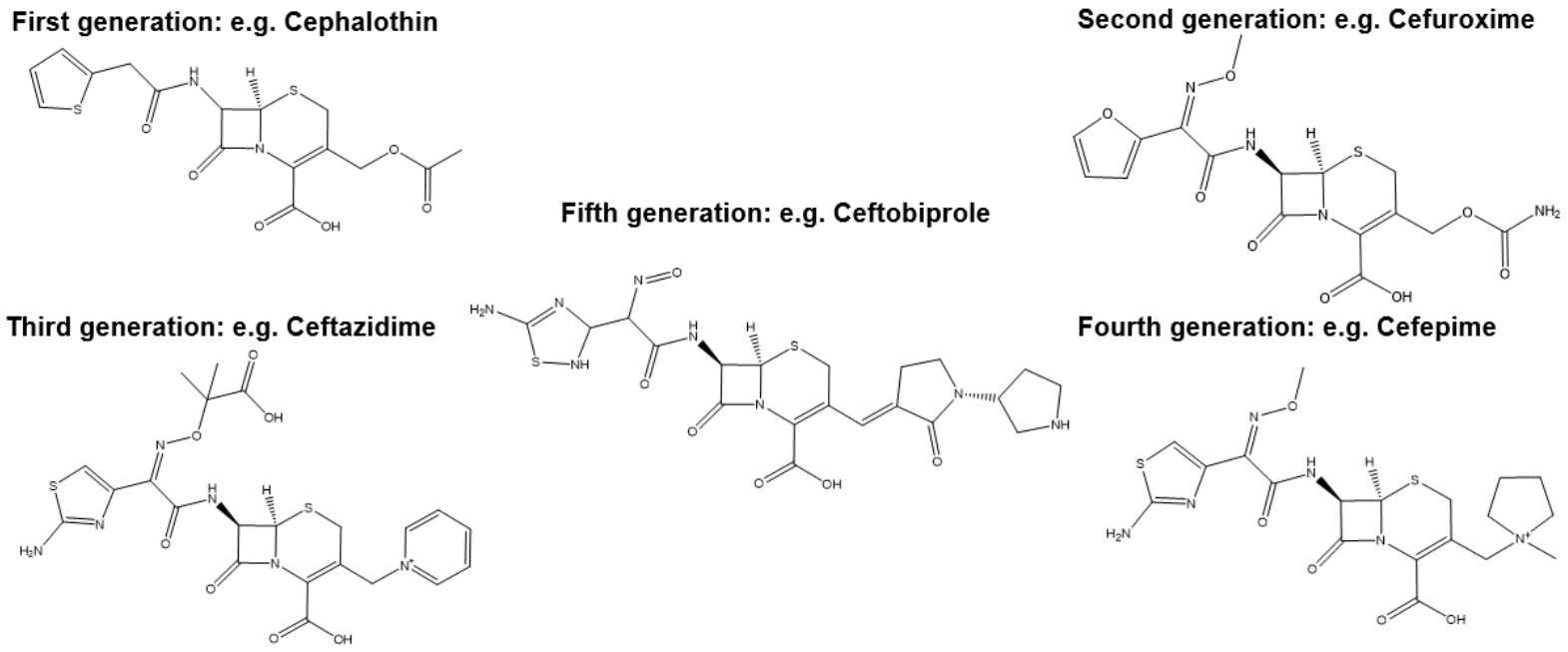

Figure 5. Chemical structures of first, second, third, fourth and fifth generation cephalosporins. Figure was made using ChemDraw professional 16. Again, all cephalosporins contain the β-lactam ring, this time fused to a 6-membered dihydrothiazinic ring.

1.4.2.3. Carbapenems

Carbapenems show the most potent activity of all the sub groups within the β-lactam antibiotic class (67). Because of this, carbapenems are often labelled as 'last-resort' antibiotics for use when patients are not responding to alternative antibiotic treatments (68). Carbapenems have, not only the most potent activity, but also the broadest spectrum of activity being able to target Gram-positive, Gram-negative, including aerobic and anaerobic bacteria. It is for this reason that carbapenems remain the favoured therapy for serious multi drug-resistant infections, especially those caused by the Gram-negative bacterial species: *E. coli, K. pneumoniae* and *P. aeruginosa* (69). The first carbapenem to be discovered was thienamycin, produced by the Gram positive heterotrophic soil bacterium; *Streptomyces cattleya* (70). This bacterium is of great interest in the synthesis of new β-lactams as it is also known to produce the second-generation cephalosporin; cephamycin C as well as the first-generation penicillin; penicillin N (71). The sulphur atom in the five-membered thiazolidine ring of penicillins is replaced by a carbon atom in carbapenems. A double bond is introduced between the carbon atoms C2 and C3 and the C6/C7 acylamino group present in penicillins and cephalosporins is replaced by a hydroxyethyl group. Unfortunately, while thienamycin showed great potential *in vitro*, it could not be successfully introduced for clinical used due to its poor *in vivo* stability. This lead to a derivative of thienamycin being synthesised by the addition of a formamidine group at the C3 carbon position. Imipenem (thienamycin-formamidine) subsequently became the first clinically available carbapenem (71). However, it was quickly realised that imipenem was sensitive to hydrolysis by the human renal dehydropeptidase: DHP-1, which meant that it had to be co administered with a DHP-1 inhibitor such as betamipron or more commonly cilastatin (72, 73). To negate the need to co-administer imipenem with a dehydropeptidase inhibitor, a methyl group was introduced at the C1 position. This new carbapenem was named meropenem and was non-susceptible to hydrolysis by renal dehydropeptidase. Meropenem also contains a unique 'R' group side chain at the C2 position that greater enhances its Gram-negative bactericidal activity (figure 6) (67, 74). Carbapenems are stable against a large selection of β-lactamases, including the Ambler class A enzymes: TEM-1, SHV-1, CTX-M and the class C enzyme CMY. It is thought that this important property shown by carbapenems is due to the presence of the C1 carbon as well as the hydroxyethyl group (67).

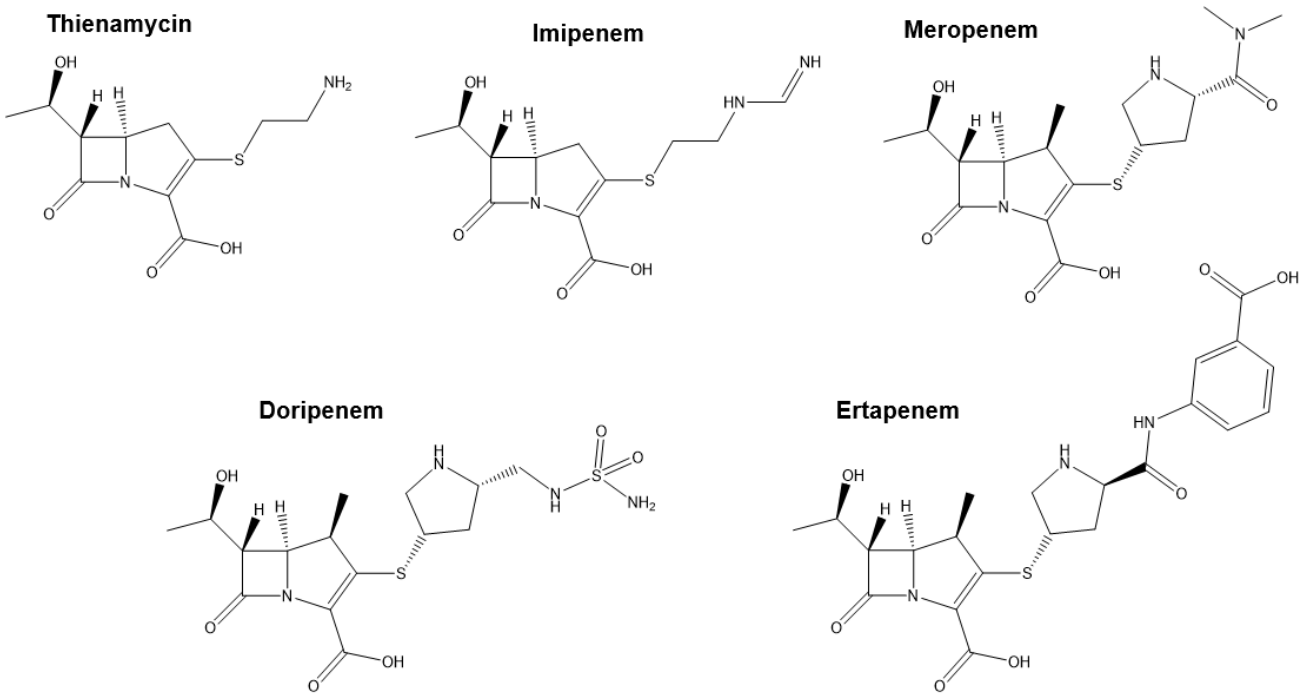

Figure 6. Chemical structure of Carbapenems. Figure was made using ChemDraw professional 16.0.

1.4.2.4. Monobactams

Like carbapenems, monobactams were originally discovered from producer bacteria, whereas penicillins and cephalosporins (with the exception of cephamycins) are produced by fungi. However, that is where the similarities to carbapenems end. The only chemical similarity monobactams have to the other members of the β-lactam class of antibiotics, is that they contain the invariable 4-membered nitrogen-containing β-lactam-ring. Unlike penicillins, cephalosporins and carbapenems, monobactams do not contain a fused ring system. Instead, monobactam derivatives contain a methoxyl group present at the C3 carbon position which improves its stability against β-lactamases. Monobactams initially showed limited Gram-negative activity and no Gram-positive activity. To try and improve this, alterations of the C3 carbon acylamido chain were introduced. Unusually, monobactams also contain the 'N-SO_3H' group, which is an incredibly rare occurrence for a natural product (figure 7) (75). The most important, and first clinically available member of the monobactam family was aztreonam. Aztreonam was discovered as a new β-lactam class in 1981 originally identified as a metabolic product of the Gram-negative soil bacterium; *Chromobacterium violaceum* (76). Aztreonam has a restricted activity and specifically targets Gram-negative

aerobic bacteria (including *P. aeruginosa*) and again has poor Gram-positive activity. However, the Gram-negative activity is comparable in its bactericidal potency to that of third generations cephalosporins (75).

Aztreonam

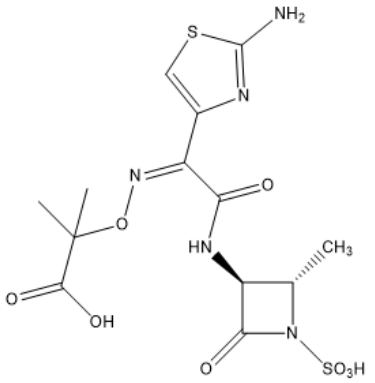

Figure 7. Chemical structure of the monobactam Aztreonam. Figure was made using ChemDraw professional 16.

1.4.3. Mechanism of action

In a bacterium, the cell wall is vital for maintaining cell shape, rigidity and osmotic stability (77). It is made of a polymer compromising of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) monomers joined by β,1-4 glycosidic bonds. However, these bonds are not enough to provide the strength required to maintain cell shape or rigidity, instead this is achieved via the trans-peptidase activity of PBPs (77, 78). It is these PBPs that are essential for bacterial cell viability. Each MurNAc monomer contains a pentapeptide: L-Ala-γ-D-Glu-L-Lys (or -meso-diaminopimelic acid in Gram-negative bacteria)-D-alanyl-D-alanine sub-unit, which serves as a cross link between the glycan polymers through PBP activity (79). It is the high molecular mass (HMM) PBPs that contain this essential trans-peptidase activity but can also have glycosyltransferase activity such is the case with class B PBPs (78, 80). The transpeptidase activity of HMM PBPs cleaves the terminal D-Ala sub-unit of the pentapeptide chain present on the MurNAc monomers. This is achieved by the formation of a Ser-D-Ala ester bond. This bond is highly reactive and can interact with the diaminopimelic acid residue of another pentapeptide chain. This results in the formation of a cross-linking peptide bond of the pentapeptide chains (81). There is also

low molecular mass (LMM) PBPs which serve to provide D, D-carboxypeptidase activity. They cleave the terminal D-alanine from the pentapeptide of MurNAc, preventing the ability of MurNAc to act as a donor for further cross linking. This D, D-carboxypeptidase activity serves as an additional layer of control in peptidoglycan synthesis. An overview of peptidoglycan synthesis is provided in figure 8 (82-84). It is the essential trans-peptidase activity of PBPs, that β-lactams can successfully inhibit. They do this by covalently binding to the active site of PBPs and subsequently inactivating the trans-peptidase activity. Specifically, it is the electrophilicity of the carbonyl group in the β-lactam that enables covalent binding and modification of the active serine residue of the PBP (85). It was first proposed by Tipper and Strominger in 1965 that β-lactams show extensive structural and steric similarity to D-Ala-D-Ala (figure 9) which is present in the MurNAc pentapeptide subunit (46). This explains the ability of β-lactams to bind to the active site of PBPs. The loss of the cross-linking activity combined with the cell-wall recycling action of peptidoglycan lytic-trans-glycosylases (see figure 10) leads to the degradation of the cell wall. This results in a build-up of osmotic pressure eventually causing the bacterial cell to burst (86).

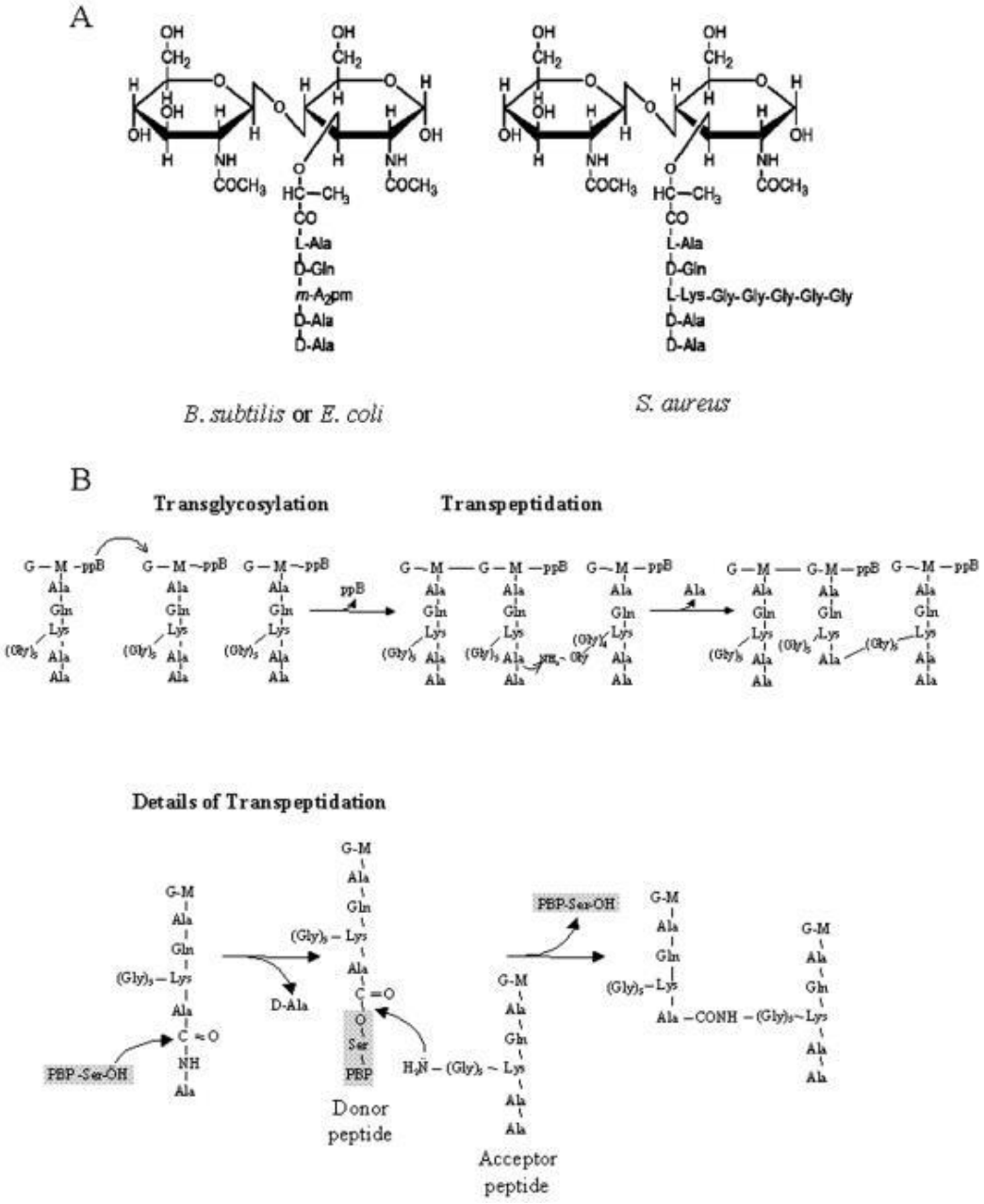

Figure 8. An overview of peptidoglycan bio-synthesis and PBP action. A) Peptidoglycan synthesis begins in the bacterial cytoplasm with the synthesis of MurNAc and GlcNAc monomers. B) These are then transported to the periplasm where they are polymerised through the action of glycosyltransferase activity of class A HMM PBPs. This results in the formation of 'immature' peptidoglycan. The pentapeptide chain present in MurNAc is then crosslinked to other MurNAc/GlcNAc polymers through the transpeptidase activity of class B PBPs resulting in the formation of 'mature' peptidoglycan. Figure taken from (87, 88).

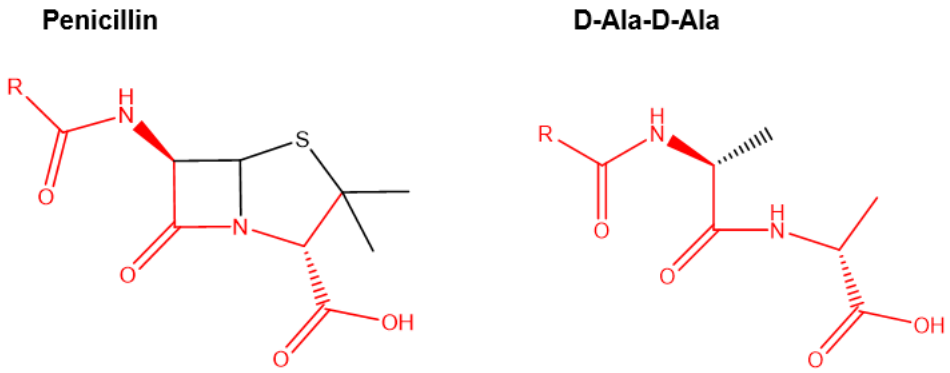

Figure 9. The steric and structural resemblance of the β-Lactam Penicillin compared to D-Ala-D-Ala, present in the pentapeptide of the peptidoglycan monomer MurNAc. Elements of the chemical highlighted in red are present in both penicillin and D-Ala-D-Ala. Figure was made using ChemDraw professional 16. Figure based on (89).

1.4.4. Cell wall recycling

The bacterial cell wall is by no means a static structure. It undergoes a continuous cycle of breakdown, followed by recycling and finally re-synthesis. It is estimated that 50% of the bacterial cell wall is turned over in one generation. During the cell wall recycling process, the majority of the peptidoglycan constituents are released back into the cytoplasm via active transport and then used to rebuild the cell wall. Alternatively, the cell wall constituents can be used as an effective energy source (90). It is this process of cell wall recycling that β-lactams interfere with. We have seen previously that β-lactams inhibit the crosslinking ability of certain PBPs. Therefore, no new peptidoglycan can be made. However, whilst β-lactams are inhibiting the synthesis of new peptidoglycan, cell wall recycling and more importantly, cell wall degradation continues to take place. Eventually it is the action of peptidoglycan-lytic-transglycosylases that lead to the complete breakdown of the cell wall and consequently the death of the bacterial cell. A summary of the bacterial cell wall recycling process is provided in figure 10. While bacterial cell wall recycling is important for recovering resources, it can also serve as a mechanism for the recognition of cell wall acting antibiotics such as β-lactams. This recognition allows the bacterium to use the cell wall recycling process as a switch to turn on intrinsic resistance mechanisms, such as the production of the AmpC β-lactamase (figure 10) (89-91).

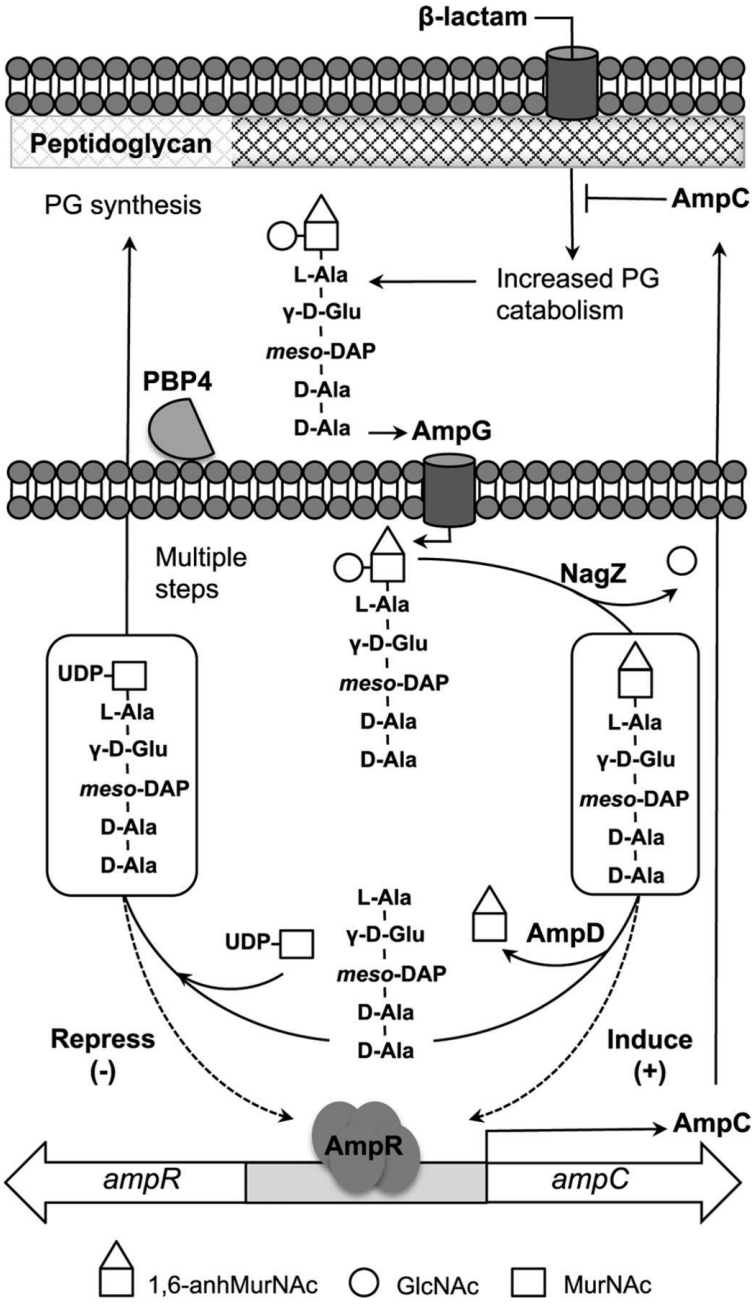

Figure 10. An overview of cell-wall recycling and its link with β-lactamase production. Bacterial cell wall recycling is initiated in the periplasm by the hydrolysis of MurNAc/ GlcNAc polymers through the action of peptidoglycan lytic transglycosylases (e.g. Slt70). This results in the formation of MurNAc/GlcNAc peptides which are actively pumped in to the cytoplasm for recycling by the action of the AmpG permease (92, 93). NagZ, a glucosaminidase, removes the GlcNAc sugar residue to produce 1,6 anhydro-MurNac peptides (serve as AmpR activators) (94, 95). The amidase AmpD can then cleave these molecules to form 1,6 anhydro-MurNac, which can re-enter the peptidoglycan biosynthesis pathway through an Mpl dependant-catalysed reaction to form UDP- MurNAc pentapeptides (AmpR repressors) (90, 96). The balance between peptidoglycan synthesis and breakdown therefore dictates

the balance between AmpR activator and repressor ligand concentration, and so the activity of AmpR and transcription levels of the *ampC* β-lactamase gene. Figure taken from (96).

1.5. Antibiotic resistance against β-lactams

Resistance to β-lactams can occur through four main mechanisms. The first, and by far the most clinically significant, is the hydrolysis of β-lactams through the action of β-lactamase enzymes (an in-depth overview of β-lactamases is provided in section 1.6.) (89). It is approximated that the global financial impact of β-lactamase producing bacteria is in excess of $30 billion per year (97). The second resistance mechanism is due to the presence of target site modifications. This is where the target of the antibiotic is altered, through mutation, so that the antibiotic is no longer, or is less able to bind. Importantly, these mutations must be sufficient to avoid the action of the drug but also maintain their function. This particular mechanism is rare in Gram-negative bacteria but is important in Gram-positive bacteria such as *S. pneumoniae* and also in *Mycobacterium tuberculosis* (98). The third is the acquisition of a non-susceptible target such is the case with Methicillin-resistant *S. aureus* (MRSA). The final mechanism of β-lactam resistance is achieved by altering the bacterial cell permeability, either by blocking entry through porins and/or through active removal using efflux pumps. Frequently, more than one mechanism is present at the same time in the same bacterium.

1.5.1. Acquisition of a non-susceptible target

We have already seen the essential role that high-molecular mass PBPs play with regards to cellular rigidity and osmotic stability of the bacterial cell. Therefore, organisms cannot evade β-lactam action by simply discarding PBPs. However, mutation of PBPs to reduce binding and or acylation by β-lactams is possible. Perhaps the most famous example of the acquisition of a non-susceptible target is the acquisition of the *mecA* gene in *S. aureus* (99). The *mecA* gene was first identified in 1985 and was found to be the resistant determinant for MRSA (100). The *mecA* gene expresses a novel PBP that became commonly referred to as PBP2a or PBP2'. It is hypothesised that the *mecA* gene originated in the animal pathogen *Staphylococcus sciuri* and mobilised onto the staphylococcal cassette chromosome *mec* (SCC*mec*) allowing horizontal transfer between different *Staphylococcus spp.* (101, 102). β-lactams interact with PBPs by forming a stable covalent complex which subsequently allows acylation of the catalytic serine residue of the PBP. Through kinetic studies it has been found

that the reduced sensitivity of PBP2a to β-lactams is due to a reduced acylation rate constant, resulting in a failure to form the initial non-covalent complex (103). The impact of *Staphylococcus spp.* carrying the *mecA* gene, is that they become resistant to Methicillin and many other β-lactams. However, several β-lactams have been identified that retain activity against the novel PBP2a. These are synthetic cephalosporins and carbapenems that are currently in different stages of development and display a specifically high affinity for PBP2a (104) (99).

1.5.2. Target-site modification

Organisms such as the Gram-positive *S. pneumoniae* achieve β-lactam resistance through alteration of their PBPs. *Pneumococci* such as *S. pneumoniae* possess five HMM PBPs which are thought to be of significant importance with regards to β-lactam resistance: PBP1A, PBP1B, PBP2A, PBP2B and PBP2X. They also contain a LMM PBP, PBP3 which is not thought to have an implication in β-lactam resistance (105).

The alterations to these PBPs results in a greatly decreased affinity for many β-lactams, including third generation cephalosporins (106). These PBPs that have a low affinity for β-lactams are encoded by mosaic genes. Mosaic PBPs are achieved by which part of the original PBP sequence is replaced by allelic variants in patches along the gene. These mosaic PBPs can differ in their sequence by up to 25% (107, 108). The origin of these patches of variation is thought to have occurred through horizontal gene transfer from non-pathogenic commensal species (109). Mosaic PBPs are also an important β-lactam resistant determinant in other naturally transformable bacteria such as *H. influenzae* (110).

1.5.3. Outer Membrane Permeability

The outer membrane of Gram-negative bacteria provides an additional layer of defence against antibiotics. The membrane acts as a discriminatory barrier, restricting the entry of toxic substances and antibiotics. However, while the gram-negative outer membrane is notably impermeable, nutrients must still be allowed to enter the cell. They enter through outer membrane proteins called porins. Porins are substrate-selective channels that facilitate the entry of hydrophilic molecules less than 600 Da into the cell (111). Some hydrophilic antibiotics, such as β-lactams can also pass through these porins into the periplasmic space, while hydrophobic antibiotics (e.g. aminoglycosides and macrolides) use a lipid mediated pathway through the outer membrane (112). Porins, and their impact on antibiotic resistance

has been well documented. The production of porins is often downregulated or modified to limit the entry of the antibiotic and therefore protect the antibiotic target. The downregulation of porins has been described in a wide selection of organisms including, but not limited to: *E. coli, P. aeruginosa, K. pneumoniae* and *Enterobacter aerogenes* (113). Many studies on porins are focused on OmpF and OmpC of *E. coli* because of the presence of almost identical proteins in many Gram-negative enteric species, for example *K. pneumoniae* contains OmpK35 (an OmpF homolog) and OmpK36 (an OmpC homolog) (114, 115). A good example of the role or porin downregulation in antibiotic resistance is provided by resistance to the carbapenem ertapenem in *K. pneumoniae.* A CTX-M expressing clinical isolate was found to be ertapenem resistant, even though CTX-M is not considered to be a carbapenemase. It was found that the isolate was down-regulating the OmpK36 porin and that ertapenem sensitivity could be restored, via the introduction of an OmpK36 expressing plasmid (116). In another analysis, surveying of antibiotic resistant *E aerogenes* in a hospital found that 44% of 45 β-lactam resistant isolates, had reduced production of porins (117). For a more detailed overview of outer membrane permeability in *K. pneumoniae*, see section 1.7.3.

1.5.4. Efflux pumps

Efflux pumps are present in both Gram-positive and Gram-negative bacteria and are located on the cytoplasmic membrane. Their genes are commonly found on the bacterial chromosome, though they have been found on mobile genetic elements. Efflux pumps are transport systems involved in the active expulsion of toxic substances (including antibiotics) (118). Efflux pumps can be divided in to five major families, these are: Major facilitator superfamily (MFS), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP binding cassette (ABC). ABC efflux pumps use ATP-hydrolysis to provide the energy needed to extrude toxic substrates, whereas all remaining efflux families exploit proton motive force as an their energy source (119). Efflux pumps can either be substrate specific or are able to expel multiple substrates. Many antibiotic classes, including β-lactams are pumps (efflux pumps that are capable of conferring resistance to three or more classes of antibacterials are described as displaying a multi-drug resistance phenotype (MDR)). Therefore, it should be of no surprise that over-production of efflux-pumps is seen as a resistance mechanism in both Gram-positive and negative bacteria. Over-production of efflux pumps has been described in several clinically important bacterial species including: AcrAB-TolC of *E. coli* and *K. pneumoniae,* MexAB-OprM of *P. aeruginosa* to name but two (120). AcrAB-TolC and MexAB-OprM are members

of the RND family tripartite efflux pumps whose substrates include β-lactams. Although overproduction of these efflux pumps is thought to provide a role in acquired β-lactam resistance, resistance is usually a result of effluent pumps alone (120, 121). More commonly, over-production of efflux pumps is combined with the production of a β-lactamases or down-regulation of porins or both.

1.6. β-lactamases

1.6.1. Overview

The most common mechanism of β-lactam resistance in Gram-negative bacteria is the hydrolysis of the antibiotic by β-lactamases (122) (123). Although the production of β-lactamases is not the prominent resistant mechanism in Gram positive bacteria, it still poses a large threat in bacteria such as *S. aureus* (124). β-lactamases are responsible for hydrolysis of the β-lactam ring, subsequently inactivating the β-lactams' bactericidal activity. The resulting product from β-lactam hydrolysis is inactive β-amino acids (35). Without the action of the β-lactam, the bacterium retains its transpeptidase cross-linking activity, allowing the cell wall to maintain its rigidity and prevent lysis. So far, in excess of 200 types of β-lactamases have been discovered(89). β-lactamases carry out their function in the bacterial periplasm, which is also the location of β-lactam action and can either be carried on plasmids, transposons or intrinsically encoded on the bacterial chromosome (125). They are classified either according to the Ambler classification system based on their amino acid sequence and hydrolysis mechanism (123) or according to the Bush-Jacoby-Medeiros group system according to their functionality and substrate specificity (table 1) (126). Concerningly, all classes of β-lactamases can be found in the large Gram-negative family of Enterobacteriaceae which include the clinically relevant pathogens *E. coli* and *K. pneumoniae* (127). The increased prevalence of β-lactamases has followed the development and increased consumption of new β-lactams. There are four distinct examples of this. The first was the dissemination of penicillinases in response to the use of aminopenicillins for treating Gram-negative infections. Penicillinases included the Ambler class A TEM-1 and SHV-1 enzymes, which show high potency against penicillins but a narrow spectrum of activity against cephalosporins (128). The second example was the mutation of TEM-1 and SHV-1 selected for by the increased usage of cephalosporins. These mutations enabled them to extend their β-lactam hydrolytic profile to include cephalosporins. This increase in activity to allow hydrolysis of larger substrates lend to these enzymes being referred to as extended spectrum β-lactamases (ESBLs) (129, 130). The third example was

the unexpected dissemination of the class A ESBL CTX-M, which became mobilised from the chromosome of the environmental bacterial species *Kluyvera* spp. (see section 1.9.2.1.) (131). The last and most recent example was the dissemination of enzymes capable of hydrolysing carbapenems. These carbapenem hydrolysing enzymes (carbapenemases) include the Ambler class A ***K****. **p**neumoniae* **c**arbapenemase (KPC) (132), the Ambler class B metallo- β-lactamases: IMP, VIM, NDM. And finally, a variety of Ambler class D oxacillinases (133, 134).

Ambler class	Bush-Jacoby Medeiros group	Active site	Enzyme type	Host organisms	Substrates
A	2b, 2be, 2br, 2c, 2e, 2f	Serine	Broad-spectrum β-lactamases (TEM, SHV)	Enterobacteriaceae and nonfermenters	Ampicillin, cephalothin
			ESBL (TEM, SHV, CTX-M)		Penicillins, 3rd-generation cephalosporins
			Carbapenemases (KPC, GES, SME)		All β-lactams
B	3	Zinc-binding thiol group	Carbapenemases (VIM, IMP)	Enterobacteriaceae and nonfermenters	All β-lactams
C	1	Serine	AmpC cephamycinases (AmpC)	*Enterobacter* species *Citrobacter* species	Cephamycins, 3rd-generation cephalosporins
			AmpC cephamycinases (CMY, DHA, MOX FOX, ACC)	Enterobacteriaceae	Cephamycins, 3rd-generation cephalosporins
D	2d	Serine	Broad-spectrum β-lactamases (OXA) ESBL (OXA)	Enterobacteriaceae and nonfermenters	Oxacillin, ampicillin, cephalothin
			Carbapenemases (OXA)		Penicillins, 3rd-generation cephalosporins All β-lactams

Table 1. β-Lactamase class and a description of enzyme type, host and substrate specificity. The Ambler class system divides β-lactamases into four distinct families: A, B, C and D. Ambler class A β-Lactamases contain penicillinases: TEM and SHV, ESBLS such as CTX-M and finally, carbapenemases such as KPC and SME. Ambler class C β-lactamases include the chromosomally encoded, cephalosporinase AmpC. Ambler class D β-lactamases contain the oxacillinases OXA of which some are carbapenemases. Ambler class A, C and D are all **s**erine **β**-**l**actamases (SBLs), so named due to the serine catalytic active site. The serine residue of SBLs works by becoming self-acylated following the hydrolysis of the β-lactam ring. SBLs show structural and steric similarity to PBPs, which explains their ability to bind β-lactams. However, Ambler class B β-lactamases show little structural similarity to SBLs and instead work though a zinc-dependant active site that requires water molecules to carry out β-Lactam hydrolysis. Ambler class B β-lactamases are referred to as **m**etallo- **β**-**l**actamases or MBLs due to their zinc-dependency in their active site (123). MBLs can be divided in to three sub-classes; B1, B2 and B3, depending on the amino acid sequence, number of zinc ions and coordinate water molecules present in the active site (135, 136). Figure adapted from (127).

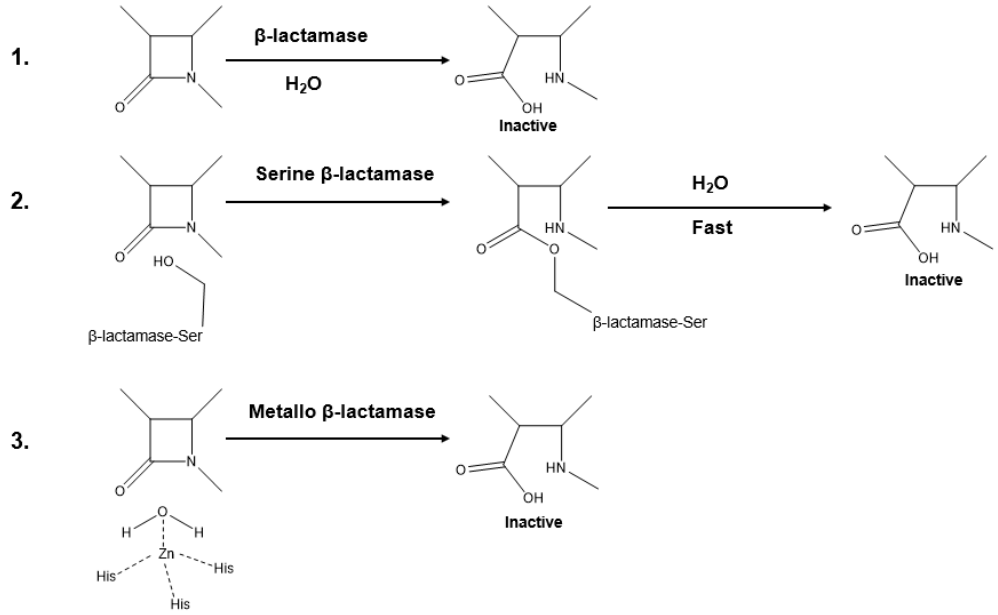

Figure 11. Structural representation of β-lactam hydrolysis by β-lactamases. Figure was made using ChemDraw professional 16. 1. Overall hydrolysis mechanism common to all β-lactamases. 2. Mechanism of β-lactam hydrolysis by Serine- β-lactamases. SBLs utilise a serine residue to hydrolyse the β-lactam ring. They subsequently utilise a water molecule to de-acylate the acyl-enzyme intermediate allowing recycling of the enzyme 3. Mechanism of β-lactam hydrolysis by MBLs. MBLs utilise a metal ion, in this case zinc, to co-ordinate a hydroxide to attack the β-lactam ring.

1.6.2. Serine -β-lactamase mechanism of hydrolysis

As mentioned previously, SBLs are thought to be evolved from PBPs. Similarly, to PBPs, SBLs utilise a serine residue to hydrolyse β-lactams. However, they differ to PBPs in that they possess the ability to exploit a water molecule to subsequently de-acylate the acyl-enzyme intermediate (figure 11). This allows the enzyme to be recycled and therefore able to be present for subsequent hydrolysis reactions. All SBLs contain an SXXK motif whereby the serine is the active residue, used as a nucleophile. Deprotonation of this serine residue using a general base creates a potent nucleophile which can subsequently carry out a nucleophilic attack on the β-lactam ring, resulting in the formation of an acyl-enzyme intermediate (figure 11). The acyl-enzyme intermediate is then resolved by the use of a water molecule previously activated by the general base. The three different ambler classes of SBLs (A, C and D) differ in the exact detail of their hydrolysis mechanism as they utilise different general bases, responsible for the acylation and de-acylation reactions.

1.6.2.1. Class A

It is widely accepted the class A SBLs utilise the Glu166 residue as the general base, which carries out the deprotonation of the water molecule allowing de-acylation of the acyl-enzyme intermediate. However, the exact identity of the general base responsible for deprotonation of catalytic serine residue remains a more contentious topic. One hypothesis states that Glu166 is also involved in the acylation reaction, whereby it deprotonates a water molecule which can subsequently deprotonate the catalytic Ser70. Alternative theories state the involvement of the Lys73 residue and a combinative mechanism using both Glu166 and Lys73 (137). However, an NMR analysis of the TEM-1 enzyme showed that the Lys73 residue had a pKA of >10 meaning that it is unlikely that this could act as a proton acceptor to allow the deprotonation of the Ser70 (138). Class A SBLs contain an Arg244 residue that is responsible for the sensitivity to classical β-lactamase inhibitors such as clavulanic acid (see section 1.6.1.) (139).

1.6.2.2. Class C

Similarly, to class A SBLs, the mechanism of hydrolysis by class C SBLs remains poorly understood. There is a clear lack of an equivalent Glu166 in class C SBLs to facilitate the de-acylation process. It is theorised that the Tyr150 residue could potentially act as the general base for this reaction. The protonation of Tyr150 has been observed in an analysis of the structure of AmpC, thus supporting the theory that Tyr150 could act as a proton acceptor (140, 141). Recently, the amino acid residue responsible for the acylation reaction has been elucidated. This is the Lys67 residue. It is also thought that Ser64, Asn152, and Ala220 play a role in the protonation of Tyr150 and Lys 67 (142). Class C SBLs lack the Arg244 residue that is present in class A SBLs. This means that class A SBLs are insusceptible to β-lactamase inhibitors such as clavulanic acid (139).

1.6.2.3. Class D

The mechanism of hydrolysis by class D SBLs is far better understood. The presence of carbamylated Lys70 residue is thought to provide the deprotonation potential for both the activation of the catalytic Ser67 for the acylation of the β-lactam, but also the water molecule allowing de-acylation of the enzyme (143, 144). Again, class D SBLs do not contain an equivalent Arg244 residue meaning they are insensitive to β-lactamase inhibitors such as

clavulanic acid. However, they are sensitive to reversible non-β-lactam β-lactamase inhibitors such as avibactam (see section 1.5.3.2.) (145).

1.6.3. Metallo- β-lactamases mechanism of hydrolysis

The first identified MBL was the chromosomally encoded enzyme BcII in 1967, produced by the Gram-positive bacterium *Bacillus cereus* (146). They differ to SBLs not only due to their metal ion dependency but also their hydrolytic mechanism. Their substrate profile includes penicillins, cephalosporins and carbapenems (147). MBLs can be found as chromosomally encoded enzymes such as L1 of *Stenotrophomonas maltophilia* or mobilised on plasmids such as the IMP, VIM and NDM enzymes and can therefore be found in many bacterial species. MBLs are of clinical importance, as they are able to confer carbapenem resistance (148).

All MBLs contain an αββα motif which contains a metal ion binding site in the β sandwich (149). There are three distinct sub classes of MBLs, that differ by the number of metal ions in their catalytic site, but also their hydrolytic mechanisms. Class B1 MBLs usually contain two zinc ions locate in the Zn1 and Zn2 sites. The coordination of these metal ions is completed by two water molecules, one of which bridges the two metal ions whereas the other is bound to the Zn2 zinc ion. It is thought that the Zn1 zinc ion is responsible for the catalytic activity, however this is heavily debated (150). The substrate profile of class B1 MBLs includes penicillins, cephalosporins and carbapenems. Class B2 MBLs utilise a single zinc ion bound in the Zn2 site to activate the nucleophilic water molecule (151, 152). The catalytic domain is comparatively narrower than that of class B1 MBLs. This is theorised to be the reason why class B2 MBLs have a limited substrate specificity directed mainly at carbapenems (153). The hydrolytic mechanisms of class 3 MBLs remains relatively poorly understood compared to the other classes. It is thought that class 3 MBLs utilise two zinc ions located at the Zn1 and Zn2 sites, however the zinc ion coordination differs to that of class 1 MBLs (136). Class 3 MBLs are able to hydrolyse penicillins, cephalosporins and carbapenems, with reduced efficiency against carbapenems compared to class 1 and 2 MBLs (153). All classes of MBLs remain sensitive to monobactams (154).

1.7. β-lactamase Inhibitors

As a result of the continual consumption and prescription of β-lactams, the selection pressure on β-lactamase carrying bacteria will only continue to increase. This is of greatest concern in Gram-negative Enterobacteriaceae family, whereby *E. coli* and *K. pneumoniae* are the most important members. It is also of concern in bacteria belonging to the non-fermenting Gram-negative bacilli: *S. maltophilia, A. baumannii* and *P. aeruginosa* (155). We are currently reaching a dire situation in which these Gram-negative pathogens are becoming resistant to last resort antibiotics such as carbapenems. Promisingly, β-lactamase inhibitors have been discovered and as the name suggests, inhibit the β-lactamase hydrolytic activity, restoring the sensitivity and bactericidal activity to β-lactams used in combination with them. So far, several class A β-lactamase inhibitors are available for clinical use including clavulanic acid, tazobactam and sulbactam. More recently avibactam and vaborbactam have been licensed for clinical use and have activity against class A, C, and D and class A and C β-lactamases, respectively (156, 157).

1.7.1. Classical β-lactamase inhibitors

Since the discovery of β-lactamases in the 1940s efforts soon became focused on finding compounds capable of inhibiting these enzymes (158). However, it was not until the early 1970s that this yielded any success. The discovery of the olivanic acids, which included carbapenems and clavulanic acid, in 1976 heralded the first example of a compound capable of inhibiting β-lactamases (159). These compounds where isolated from *Streptomyces spp.* Carbapenems where shown to have potent antibacterial activity, but clavulanic acid, conversely had a very poor antibacterial activity. Interestingly though, clavulanic acid was shown to have impressive inhibitory activity as it was able to reduce ampicillin minimal inhibitory concentrations (MICs) against a diverse range of β-Lactamase producing *E. coli* and *S. aureus*. Since then several β-lactam related inhibitors have been discovered including the penicillin sulfones: sulbactam and tazobactam (158, 160, 161).

Clavulanic acid, tazobactam and sulbactam are all ‘classical’ β-lactamase inhibitors so named due to their structural similarity to the β-lactam penicillin. They function by forming a stable, irreversible covalent attachment to the serine active site present in class A β-lactamases. Consequently, the total hydrolytic activity in the cell is reduced to near zero. The activity of classical β-lactamase inhibitors against class A ESBLs is well documented, however, they have been shown to have very poor activity against the class A

carbapenemase KPC and even induce the production of the chromosomally encoded class C β-Lactamase AmpC, which they cannot inhibit (162, 163). To thwart β-lactam resistance caused by AmpC induction, and to improve activity against the KPC β-Lactamase, two non-classical class A/C β-lactamase inhibitors have been developed and licensed for clinical use: Avibactam and vaborbactam (164, 165).

1.7.2. Avibactam

Avibactam was the first 'non-classical' or 'non-β-lactam β-lactamase inhibitor' used clinically. Synthesised in 2012, it was designed to contain a diazabicyclo-octane cyclic core (164). It was made clinically available in 2014 in combination with the third-generation cephalosporin ceftazidime, trading under the name 'AvyCaz'. In vitro, avibactam shows good activity against many class A (TEM-1, SHV-1, CTX-M-15), C (AmpC) and even some class D (OXA-24 and OXA-48) β-lactamases. Impressively, it also shows inhibitory activity against the class A carbapenemase KPC (166, 167).

Avibactam has a novel inhibitory mechanism by having structural similarity to the electrophilic carbonyl group present in the serine active site of β-lactamases. Therefore, it functions by forming a reversible interaction with the serine active site, subsequently inhibiting the β-lactamase's hydrolytic activity (164). Interestingly, although it has been shown to have activity *in vitro* against the class A carbapenemase KPC, when used *in vivo*, resistance to ceftazidime/avibactam developed quickly. This was reported as being caused by a series of point mutations in the *blaKPC* gene resulting in a change in amino acid sequence: a D179Y/T243M double substitution, or single substitutions in D179Y or V240G. These changes each resulted in a 4-fold increase in MIC against ceftazidime when put in combination with the standard concentration of avibactam (168). This rapid emergence of ceftazidime/avibactam resistance in KPC producing bacteria, highlighted the need for an alternative class KPC inhibitor. A similar mechanism of ceftazidime/avibactam resistance was seen with the class A cephalosporinase CTX-M. Two single nucleotide polymorphisms were identified in the *blaCTX-M* gene leading to a double amino acid substitution, P170S and T264I. These two amino acid changes collectively gave rise to a novel derivative of CTX-M-14 that had increased ceftazidime hydrolytic activity and so increased ceftazidime MICs in the presence of a fixed concentration of avibactam (169).

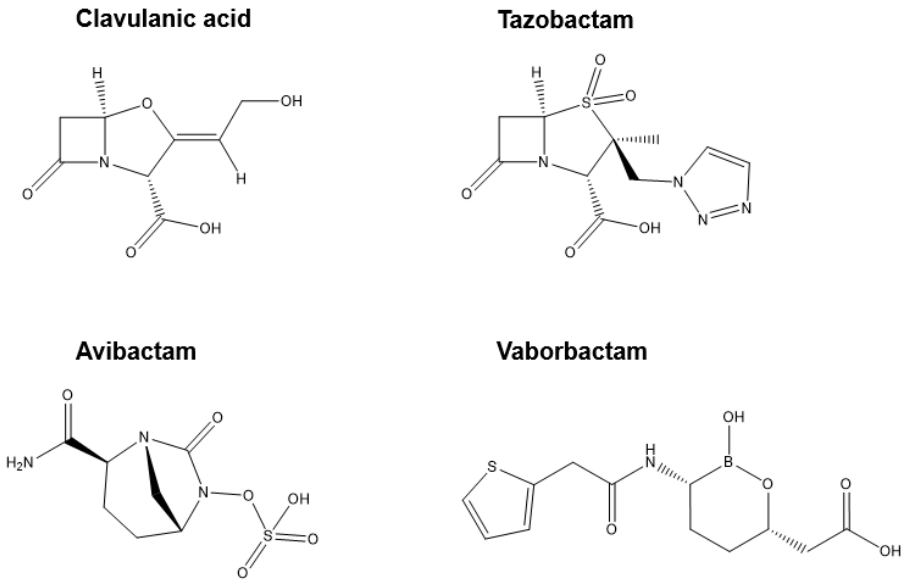

Figure 12. β-lactamase inhibitor structures. Figure was made using ChemDraw professional 16.0. Clavulanic acid and Tazobactam are examples of so called classical β-lactamase inhibitors, as they have significant resemblance to the structure of penicillins. Both clavulanic acid and tazobactam contain the 4-membered nitrogen based β-lactam ring. Avibactam and vaborbactam are non-β-lactam β-lactamase inhibitors, which contain a diazabicyclo-octane cyclic core and a cyclic boronic acid core respectively.

1.8. Vaborbactam

1.8.1. Overview

Vaborbactam is another non-β-lactam β-lactamase inhibitor, first synthesised in 2015. It was licensed for clinical use in 2017, in combination with the carbapenem meropenem and has since been trading under the name ‘Vabomere’. It was discovered during a screen specifically looking for inhibitors capable of inhibiting KPC carbapenemases (165). Therefore, it is of no surprise that it has potent activity against many class A carbapenemases including KPC variants as well as NMC-A and SME. It has also been shown to have inhibitory activity against other class A enzymes (TEM, SHV and CTX-M) and class C β-lactamases (AmpC), although to a lesser extent when compared to its activity against KPC (170). Unfortunately, vaborbactam is unable to inhibit class B and D β-lactamases. Vaborbactam has been shown to restore meropenem sensitivity in KPC-producing *K. pneumoniae* and *E. coli*, but does not restore meropenem sensitivity in non-fermenting Gram-negative bacilli (171). Vaborbactam contains a cyclic boronic acid core

(figure 12). It has been known for nearly 50 years that boronic acids are capable of inhibiting serine proteases. It is the strong electrophilic potential of the boron atom that means it has a high tendency to form covalent bonds with enzymes that contain serine catalytic active sites (172). vaborbactam is therefore able to form reversible covalent bonds with β-lactamases. Because this reaction is reversible, vaborbactam is not hydrolysed and rendered inactive during the reaction. This allows vaborbactam to be continuously recycled to inhibit other β-lactamase enzymes (165). Avibactam also works by forming reversible covalent bonds. The exception of this is with KPC, which can slowly convert avibactam in to inactive fragments (145). It is for this reason combined with point mutations in the *blaKPC* gene seen in avibactam-ceftazidime resistant mutants, that makes vaborbactam the β-lactamase inhibitor of choice with regards to KPC carrying strains (173). Vaborbactam and meropenem are both known to enter *K. pneumoniae* through the OmpK35 (OmpF) and OmpK36 (OmpC) porins. Therefore, resistance to meropenem/vaborbactam has been observed due to loss of function mutations in *ompC* and *ompF*, inhibiting the entry of the both vaborbactam and meropenem (170, 174). Increased efflux and increased KPC production have also been described as a determinant for vaborbactam resistance. More specifically, increased KPC production has been linked to the transfer of the Tn*4401* transposon (carrying *blaKPC*) from the pKpQIL plasmid, which has a low-copy number, to the comparatively higher-copy number plasmid ColEST258 as well as to *bla*KPC duplication events (174). As of yet, mutations in the *blaKPC* and *blaCTX-M* genes have not been observed that confer reduced vaborbactam inhibition. It is unclear if combinations of these resistance mechanisms are possible and whether increased vaborbactam use will lead to the development of alternative resistance mechanisms (173).

1.9. *K. pneumoniae*

1.9.1. Overview

K. pneumoniae is the most important member of the *Klebsiella* genus and belongs to the large Gram-negative family Enterobacteriaceae second only to *E. coli* in its ability to cause nosocomial infections (175) (176, 177). Such is the nosocomial ability of K. pneumoniae, that it is estimated that up to 8% of all nosocomial infections in the US and Europe annually, are caused by *K. pneumoniae* (175). Increasingly, it is an MDR human pathogen that can be found as a normal member of the mouth, skin, lung and intestinal flora, but commonly causes infections in patients that are already immunocompromised. The most common infection associated with *K. pneumoniae* is pneumonia which frequently presents in the form

of bronchitis closely followed by bacteraemia. In addition to pneumonia, it can cause a notably broad range of infections, including: wound infections, urinary tract infections (UTIs), diarrhoea, osteomyelitis and even meningitis (175). More recently other members of the *Klebsiella* genus including *Klebsiella oxytoca* and *Klebsiella rhinoscleromatis* are becoming increasingly prevalent as causes of nosocomial infections (178). But it is *K. pneumoniae* that is responsible for the most cases as it is estimated that in the UK alone, *K. pneumoniae* is responsible for 9617 (as of March 2018) cases of bacteraemia's annually, with 2853 of these being hospital acquired and over 2000 isolates showing MDR (179, 180). Since 2017, it has become mandatory to report bacteraemia caused by *K. pneumoniae*, such is the threat that it poses. *K. pneumoniae* has very quickly become a major threat due to its ability to gain resistance to multiple classes of antibiotics. However, it is *K. pneumoniae* strains resistant to carbapenems, which is of greatest concern. These strains that show carbapenem resistance are referred to as carbapenem resistant Enterobacteriaceae (CRE) (181).

1.9.2. Antibiotic resistance in *K. pneumoniae*

K. pneumoniae that are cephalosporin and/or carbapenem resistant are top on the World Health Organisations (WHO) list of critically resistant pathogens (182). Critically resistant pathogens are often referred to as ESKAPE pathogens: ***E****nterococcus faecium,* ***S****. aureus,* ***K.*** *pneumoniae ,* ***A****cinetobacter baumannii,* ***P.*** *aeruginosa* and ***E****nterobacter spp.* Together these pathogens contribute to the majority of nosocomial infections as they are able to escape the action of multiple antibiotic classes due to their frequently having an MDR phenotype (183). *K. pneumoniae* can become resistant due to its ability to increase the production of two MDR, RND efflux pumps (figure 13) as well as being able to reduce porin production. Loss of the porin OmpK36 has been well documented in reduce carbapenem susceptibility (184). But it is the loss of OmpK35 and OmpK36 porins and the presence of a β-lactamase such as CTX-M that is usually required to give carbapenem resistance (185). Undoubtedly, the biggest problem is the ability of *K. pneumoniae* to carry a wide range of plasmid acquired β-lactamases (186). The most clinically significant of which are the SBLs CTX-M (cephalosporinase), KPC (carbapenemase and cephalosporinase) and the OXA-48 like (carbapenemase). These three enzymes are the focus for this project, therefore a more detailed overview of these three enzymes is provided in sections 1.9.2.1, 1.9.2.2. and 1.9.2.3 (187-189). It has been known for some time that *K. pneumoniae* can have a MDR phenotype, with aminoglycoside and fluoroquinolone resistance being reported as early as the 1990s (177) and the first carbapenem resistance outbreak being described in 2001 (190). Currently carbapenem resistance incidence has been recorded as being as high as

54% in some studies (132).This means even last resort antibiotics are not providing a sufficient treatment for patients, leading to less effective and sometimes even sub-optimal (due to nephrotoxicity) drugs, such as colistin, being used. (191).

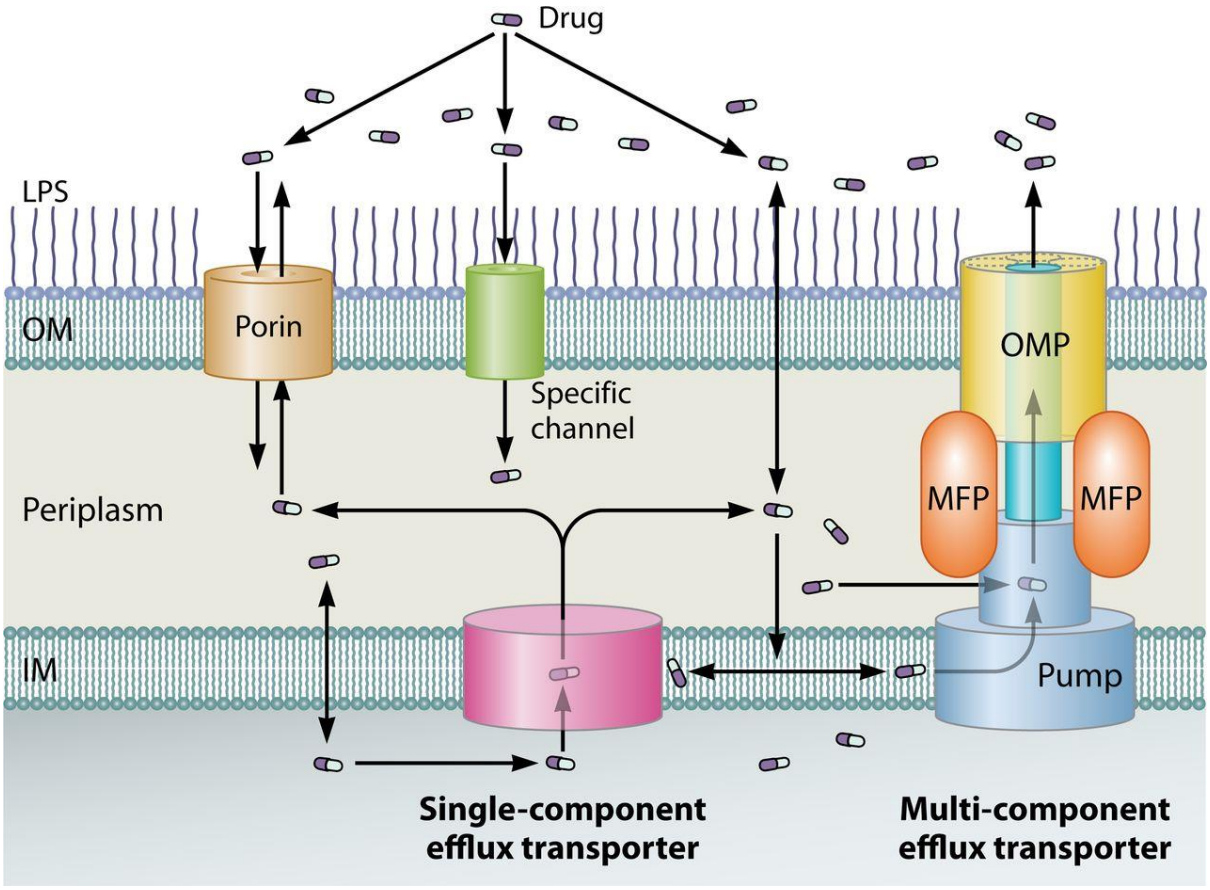

Figure 13. Diagrammatic representation of permeability and efflux components present in the Gram-negative cell wall. Tripartite or multi-component efflux pumps are shown on the which can include the families: RND, MFS and ABC. Single component efflux pumps are shown on the bottom left and may include the families: MFS, RND, ABC, MATE and SMR. In *K. pneumoniae* , the tripartite RND efflux pumps AcrAB-TolC and OqxAB-TolC are the most important indicators of ABR (192). Substrates of the RND efflux pumps present in *K. pneumoniae* include: tetracyclines, β-lactams and fluoroquinolones (193, 194). *acrAB* transcription is regulated by the production of the activator RamA, which is in turn controlled though the action of the complementary repressor RamR (belonging to the TetR family of repressors). *oqxAB* transcription is regulated by the RarA *protein* which is also under the control of a TetR family repressor, in this case OqxR. Over-expression of *ramA* or *rarA*, due to loss of function mutations in *ramR* or *oqxR,* have been shown to over express *acrAB* and

oqxAB leading to increased efflux activity and therefore an MDR phenotype (192, 195, 196). Porins indicated on the top left and facilitate the entry of a large range of antibiotic classes and other substrates. There are four key porins present in *K. pneumoniae :* OmpK35 (homologous to OmpF in *E. coli*), OmpK36 (homologous to OmpF in *E. coli*), OmpA and the inducible OmpK37 (197). However, only the porins OmpK35 (OmpF) and OmpK36 (OmpC), play an important role in ABR, as they are known to be substrates of β-lactams (198, 199). Arrows show direction of antibiotic transport in to the bacterial cell. As indicated, the antibiotic enters through porins, but then can be pumped out of the cell either by tripartite efflux pumps or into the periplasm by single component efflux pumps. Figure taken from (200, 201).

1.9.2.1. CTX-M

CTX-M is a plasmid encoded class A serine β-Lactamase (126) capable of hydrolysing a broad range of β-Lactam substrates which includes, notably, third generation cephalosporins such as cefotaxime and ceftazidime (202). The CTX-M enzyme was first discovered in 1989 (131), but did not become particularly prominent until the early 2000s, whereby an extensive dissemination of the enzyme took place, often referred to as the 'CTX-M pandemic' (203).

CTX-M enzymes can be classified into five major groups based on their amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. The origin of CTX-M is thought to have come from the chromosome of *Kluyvera spp.* , but it was subsequently mobilised by the IS*EC*p1 element on to various plasmids and it is the mobilisation of different *blaCTX-M* genes from different *Kluyvera spp.* that lead to the difference between CTX-M enzymes and ultimately the classification into five groups (204). Currently, CTX-M is incredibly common within the Enterobacteriaceae family, with a large number of bacterial species including *E. coli* and *K. pneumoniae* carrying the enzyme. In fact, it is so common that is has displaced TEM and SHV variants as the most prevalent ESBL (187).

Of all the CTX-M enzymes it is CTX-M-15 that is by far the most important. First described in 1999 in India, it has quickly spread and nowadays is found all over the world in both human, animal and environmental bacteria (202, 205). A survey carried out in Asia highlighted the high prevalence of the CTX-M-15, as 72.8% of ESBL producing *K. pneumoniae* carried this enzyme (206).

1.9.2.2. KPC

K. pneumoniae carbapenemase (KPC), is another class A serine β-lactamase (126) that has potent hydrolysing activity against penicillins, cephalosporins and most notably, carbapenems. KPC was first discovered in 2001 during a large outbreak in New York hospitals (190). The incidence of KPC carrying strains of *K. pneumoniae* was initially estimated at 9% in 2002 in the US. However, this soon rose to 33% (of carbapenem resistant infections) by 2008 (132). The first report of a KPC carrying strain of *K. pneumoniae* outside of the US was in Israel in 2004 (207). The spread of the KPC enzyme has been so successful that is now endemic in countries such as Israel and Greece and has been frequently identified in countries such as Brazil, Colombia, The United Kingdom, China and India (208).

Upon examination of the molecular epidemiology of the *blaKPC* gene it was found that the majority of isolates of carbapenem resistant *K. pneumoniae* belonged to a single sequence type; ST258 and the most common KPC variants found with in ST258 were KPC-3 and KPC-2 (208). This makes KPC-3 and -2 by far the most clinically important KPC enzymes. The reason for successful spread of KPC is largely down to its location within the transposon Tn*4401* allowing it to easily transfer between a vast number of plasmids. Because of this KPC has been found not only in *K. pneumoniae* but also several other Gram-negative bacteria including *E. coli, Proteus mirabilis, S. marcescens* and *E. aerogenes* (132).

KPC carrying isolates of *K. pneumoniae* is of great concern as there are not only financial implications associated with increased length of hospital stay. But also, treatment failure is very common and consequentially the mortality rates associated with infection of KPC expressing *K. pneumoniae* tends to be very high. Mortality rates have been reported as between 47-66% in the US and as high as 70% outside of the US (209, 210). This is further exacerbated as KPC is also often accompanied by other resistance genes, meaning isolates that carry KPC often show an MDR phenotype.

1.9.2.3. OXA-48 like carbapenemases

Unlike CTX-M and KPC, OXA enzymes are class D serine-β-lactamases. Some OXA enzymes are carbapenemases, and where first discovered in 1993 as conferring resistance to imipenem in *A. baumannii* (211). However, whereas KPC enzymes have strong carbapenemase activity, OXA enzymes have a relatively poor carbapenemase activity. Also, their activity is limited to carbapenems and penicillins and they have very little

cephalosporinase activity (212). Historically, class D serine-β-lactamases where referred to as oxacillinases due to their ability to hydrolyse **oxa**cillin, which TEM and SHV could not. It is for this reason that the term **OXA** was coined for class D serine-β-lactamases (126). OXA enzymes are varied but can be divided into many distinct groups based on amino acid sequence (134). Table 2 shows example enzymes belonging to each of the 8 clusters of OXA carbapenemase enzymes.

Cluster	**Enzyme**	**Natural host**
1	OXA-23, OXA-27, OXA-49	*A. baumannii*
2	OXA-24,25,26 and 72	*A. baumannii*
3	OXA-51	*A. baumannii*
4	OXA-58	*A. baumannii, Acinetobacter junii*
5	OXA-55, OXA-SHE	*Shewanella algae*
6	OXA-48, OXA 54	*K. pneumoniae, Shewanella oneidensis*
7	OXA-50	*P. aeruginosa*
8	OXA-60	*Ralstonia pickettii*

Table 2. Example enzymes for each OXA carbapenemase cluster and the most common host these enzymes are found in (213).

The majority of OXA carbapenemases are found in the non-enterobacterial *A. baumannii* encoded on the chromosome *(212).* However, the plasmid borne OXA-48 like enzymes can be found In *K. pneumoniae* and are therefore of clinical significance (214). As of yet, six OXA-48 variants have been described: OXA-48, OXA-163, OXA-181, OXA-204 and OXA-232 all of which differ by up to five amino acid substitutions (213). Like all OXA carbapenemases, OXA-48 like enzymes show strong hydrolysing activity against penicillins, a moderate hydrolysis of carbapenems and a very poor activity against cephalosporins. However high carbapenem MICs have been described in *K. pneumoniae* strains that produce OXA-48 like carbapenemases and also down regulate outer membrane porins (214). OXA-48 like enzymes have been found in many species including, but not limited to:

K. pneumoniae, E. coli, C. freundii and *E. cloacae.* Originally, most reports of OXA-48 carriage were limited to North African countries and Turkey (215). However, more recently OXA 48-carriage has been found across North Africa, South Asia, the Middle-East and have even spread to several European countries, notably France, Germany, Spain and the UK (216).

The main concern associated with OXA-48 producing *K. pneumoniae* is the difficulty in detecting it. Many OXA-48 producers are thought to 'slip through the net' due to the absence of a sufficient phenotypic test for recognising OXA-48 production (213). This is because OXA-48 doesn't necessarily give carbapenem resistance, rather it gives decreased carbapenem susceptibility. (217). Therefore, the impact that this has with regards to clinical outcome is very hard to decipher. Studies into the prevalence of OXA-48 like enzymes in carbapenem resistant *K. pneumoniae* suggest that it could be as high as 60% (218). It is clear that OXA-48 enzymes are of great importance with regards to a MDR phenotype in *K. pneumoniae.*

1.10. Aims of project

K. pneumoniae is undoubtedly a very important pathogen not only in its nosocomial ability but also in its ability to cause a wide variety of infections. This is exacerbated by *K. pneumoniae* strains producing β-lactamases of which KPC, CTX-M and OXA-48 like enzymes are the most clinically important. Infection with strains producing these β-lactamases will often result in β-lactam treatment failure and limit the number of viable therapeutic options to combat the infection. Promisingly, inhibitors of some or all of these enzymes have been identified, the most recent of which is the cyclic boronic acid vaborbactam licensed in combination with meropenem. MBLs, are also undoubtedly of concern in *K. pneumoniae,* but the fact that meropenem/vaborbactam is not capable of inhibiting MBLs means that this project focused on SBLs only.

It is important to characterise meropenem/vaborbactam resistance mechanisms in *K. pneumoniae* producing SBLs in an effort to aid clinical prediction of resistance and potential ways of overcoming it. Meropenem/vaborbactam resistance is known to occur but the mechanisms remain poorly understood. As mentioned previously, one study reported loss of the OmpC (OmpK36) porin as causing an increase in meropenem MICs seen in the presence of vaborbactam. However, it was also reported that several KPC strains had increased meropenem MICs without loss or reduced production of OmpC (170). It is possible that these strains with increased meropenem MICs in the presence of vaborbactam could be

a result of changes to the *blaKPC* and *blaCTX-M* genes, as seen to cause ceftazidime resistance in the presence of avibactam (167, 169). Alternatively, it could be a result of changes in permeability and efflux, not previously implemented in meropenem/vaborbactam resistance.

Therefore**, the first aim of this project was to better understand if and how permeability and efflux are involved in meropenem/vaborbactam resistance**. To achieve this aimed to test meropenem in combination with vaborbactam against a range of clinical isolates containing well defined permeability, permeability regulator, porin and efflux mutations, in the following genes: *ramR, oqxR, ompC, ompF* and also those over-expressing *micF.* More specifically, loss of function mutations in *ramR* and *oqxR* were of interest as they result in increased efflux (figure 13) (192). *micF* encodes a 93-base antisense RNA that binds and inhibits translation of *ompF* mRNA therefore reducing OmpF(OmpK35) porin production (219). Our aim was to test these mutations in an otherwise isogenic background in combination with the production of either the class A carbapenemase KPC, the class A ESBL CTX-M or the class D OXA-48 like carbapenemase OXA-232.

Secondly to confirm the previously reported importance of OmpC loss in meropenem vaborbactam **we aimed to produce *in vitro* meropenem/vaborbactam resistant mutants of clinical isolates**, again either producing CTX-M, KPC or OXA-232.

Although meropenem/vaborbactam is not a potent inhibitor of class D β-lactamases, vaborbactam activity against the class D β-lactamase OXA-2 has been reported in combination ceftazidime. Although ceftazidime/vaborbactam did not restore sensitivity it did result in decreased ceftazidime MICs in a number of different OXA producers (170). Vaborbactam has also been shown to potentiate the activity of biapenem against OXA-2,-1 and -30 *in vitro* (220). Therefore, because of this and the potential for meropenem/vaborbactam resistance, we were interested in whether alternative partners for vaborbactam could be used to target not only OXA-232 producing *K. pneumoniae*, but also KPC and CTX-M. Therefore, **the third aim of this project was to investigate potential alternative partners of vaborbactam that extend the spectrum to include OXA-232**. The alternative partners for vaborbactam tested were a selection of carbapenems (ertapenem, meropenem, imipenem and doripenem) as well as the second-generation cephalosporin cefuroxime, the third-generation cephalosporins ceftazidime, cefotaxime, ceftriaxone and the fourth-generation cephalosporin cefepime. If efficacious alternative partners of vaborbactam were to be identified, our **final aim was to identify and characterise mutants resistant to these alternative β-lactam-vaborbactam combinations**.

In summary, our aims are as follows:

1. To test a range of permeability, permeability regulator, porin and efflux mutations in an isogenic background in combination with the production of either KPC, CTX-M or OXA-232 against meropenem/vaborbactam. Our hypothesis is that loss of OmpC will cause the greatest decrease in meropenem/vaborbactam susceptibility.

2. To select for *in vitro* meropenem/vaborbactam resistant mutants of clinical isolates, again either producing CTX-M, KPC or OXA-232 and to characterise the resistant mechanism. Again, we hypothesise that loss of OmpC could be the causative mechanism as we believe that meropenem/vaborbactam resistance is actually just meropenem resistance.

3. To test a range of alternative vaborbactam partners that could extend activity to also include OXA-232. Class D β-lactamases, although capable of hydrolysing carbapenems, are relatively weak cephalosporinases. Therefore, we hypothesise that the cephalosporins tested in combination with vaborbactam could show potential.

4. To identify and characterise mutants resistant to these alternative β-lactam-vaborbactam combinations.

Chapter 2. Materials and Methods

2.1. Media

Media used throughout this project were Luria Bertani (LB) broth and agar, Mueller Hinton agar and cation adjusted Mueller Hinton broth: all purchased from Oxoid. All media was sterilised by autoclaving at 121^{o}C for 20min.

2.2. Antibiotics/ Inhibitors

Vaborbactam was purchased from MedChemExpress MCE and was solubilised in dimethyl-sulfoxide (DMSO). Avibactam was kindly supplied by Professor Schofield, Department of Chemistry, University of Oxford and was solubilised in DMSO. Both vaborbactam and avibactam were stored at -20^{O}C in powder form and -80^{O}C dissolved in DMSO. Tazobactam was obtained from Sigma-Aldrich and was solubilised in water. Tazobactam was stored at 4^{O}C in powder form and -80^{O}C dissolved in water. Antibiotic discs and bacterial growth media were supplied by Oxoid.

Antibiotic	**Manufacturer**	**Solvent**	**Class**
Kanamycin	Bio Basic Canada Inc.	Water	Aminoglycoside
Meropenem	Department of Chemistry, University of Oxford	DMSO	Carbapenem
Chloramphenicol	Sigma-Aldrich	70% Ethanol	Phenicol
Cefotaxime	Sigma-Aldrich	Water	Cephalosporin
Ampicillin	Sigma-Aldrich	Water	Penicillin
Gentamicin	Sigma-Aldrich	Water	Aminoglycoside
Doripenem	Insight Bio-Tech	DMSO	Carbapenem
Ceftazidime	Sigma Aldrich	DMSO	Cephalosporin
Piperacillin	Sigma Aldrich	Water	Ureidopenicillin
Imipenem	Insight Bio-Tech	DMSO	Carbapenem

Table 3: Companies supplying antibiotics. Stored in powder form at 4^{O}C or -20^{O}C, stored at -20^{O}C or -80 dissolved in solvent depending on stability.

2.3. Equipment

Equipment	Manufacturer
Centrifuge	ALC Multispeed Refrigerated Centrifuge PK 121R
Ultracentrifuge	Sorvall® RC 5B Plus
Plate reader	POLARstar Omega
Sonicator	Sonics Vibra cell VCX750
PCR thermal cycler	Bio Rad Peltier Thermal Cycler PTC-100
Heat Block	Eppendorf Thermomixer 5436
Vortex	Whirlmixer Fisons Scientific Equipment
Biofuge *pico*	Heraeus
Gene sys gel reader	Bio Rad Gel Doc 1000 system
Nanodrop Lite Spectrophotometer	Nanodrop Thermo Scientific
Gene Pulser	Bio Rad
Ultraspec 2100pro	Amersham Bioscience
G:BOX	Syngene

Table 4. Equipment used, and Company purchased from.

2.4. Storage and growth conditions of Bacterial strains

All strains used throughout this project, were stored at -80^{O}C contained in glycerol bead stocks (Protect Bacterial Preservers). All bacterial strains were cultured by growing overnight with 180 rpm shaking in LB broth or Mueller Hinton broth at 37^{O}C. An estimate of bacterial growth was determined by measuring absorbance (Optical density) at 600 nm (OD600).

2.5. Lab Bacterial Strains

Strain/Isolate (reference)	Species
DH5α (221)	*E. coli*
Ecl8 (222)	*K. pneumoniae*
Ecl8 44	*K. pneumoniae*
Ecl8 16-1	*K. pneumoniae*
Ecl8 0.125	*K. pneumoniae*
Ecl8 0.03-2 (223)	*K. pneumoniae*
Ecl8 0.03-1 (223)	*K. pneumoniae*
Ecl8 35 (*ΔompF)*	*K. pneumoniae*
Ecl8 36 (*ΔompC)*	*K. pneumoniae*
Ecl8 Delta (*ΔramR)* (196)	*K. pneumoniae*
Ecl8 Delta 35 (*ΔramR ΔompF)*	*K. pneumoniae*
Ecl8 Delta 36 (*ΔramR ΔompC)*	*K. pneumoniae*
Ecl8 Delta 36 micF (*ΔramR ΔompC* pK18*: micF)* (192)	*K. pneumoniae*
S17 (224)	*E. coli*

Table 5. Bacterial strains/Isolates used and their reciprocate species. KPC-3, CTX-M-15 and OXA-232 transformants of DH5α were used for plasmid purification. Strains 44, 16-1, 0.125, 0.03-2, 0.03-1, Ecl8 *ΔompF,* Ecl8 *ΔompC,* Ecl8 *ΔramR ΔompF*, Ecl8 *ΔramR ΔompC,* Ecl8 *ΔramR ΔompC* pK18*: micF* were all derived from the wild type Ecl8 strain (222). For genotypic information on these strains see table 9. S17 was used as a donor for conjugation (see section 2.12.). Knockouts were constructed by visiting PhD student Punyawee Dulyayangkul. Genotypes of these strains are provided in table 9.

2.6. Clinical isolates

Throughout this project, a library of 30 clinical isolates of *K. pneumoniae* was used, obtained from Dr Karen Bowker, Southmead Hospital, Bristol (table 6).

Isolate	Location of infection	Date Isolated
R1	Blood culture	12/04/2016
R2	Blood culture	08/03/2016
R3	Tissue	10/01/2016
R4	Tissue	07/12/2015
R5	Blood culture	05/10/2015
R6	Blood culture	25/09/2015
R7	Blood culture	01/09/2015
R8	Ascitic Fluid	23/02/2015
R9	Tissue/Kidney Stone	05/12/2014
R10	Blood culture	27/10/2014
R11	Screening Swab	10/10/2014
R12	Blood culture	01/10/2014
R13	Groin Swab	02/07/2014
R14	Wound Swab	11/06/2014
R15	Blood culture	20/05/2014
R16	Blood culture	20/04/2014
R17	Sputum	19/03/2014
R18	Catheter Urine	18/02/2014
R19	Catheter Urine	17/10/2013
R20	Sputum	01/08/2013
R21	Sputum	30/07/2013
R22	Blood culture	27/07/2013
R23	Wound Swab	23/07/2013
R24	Mid-Stream Urine	23/07/2013
R25	Blood culture	22/07/2013
R26	Mid-Stream Urine	03/07/2013
R27	Central Line Tip	17/04/2013
R28	Bronchio-alveolar Lavage	11/03/2013
R29	Blood culture	09/03/2013
R30	Blood culture	07/08/2012

Table 6. Library of *K. pneumoniae* clinical isolates.

2.7. Vectors

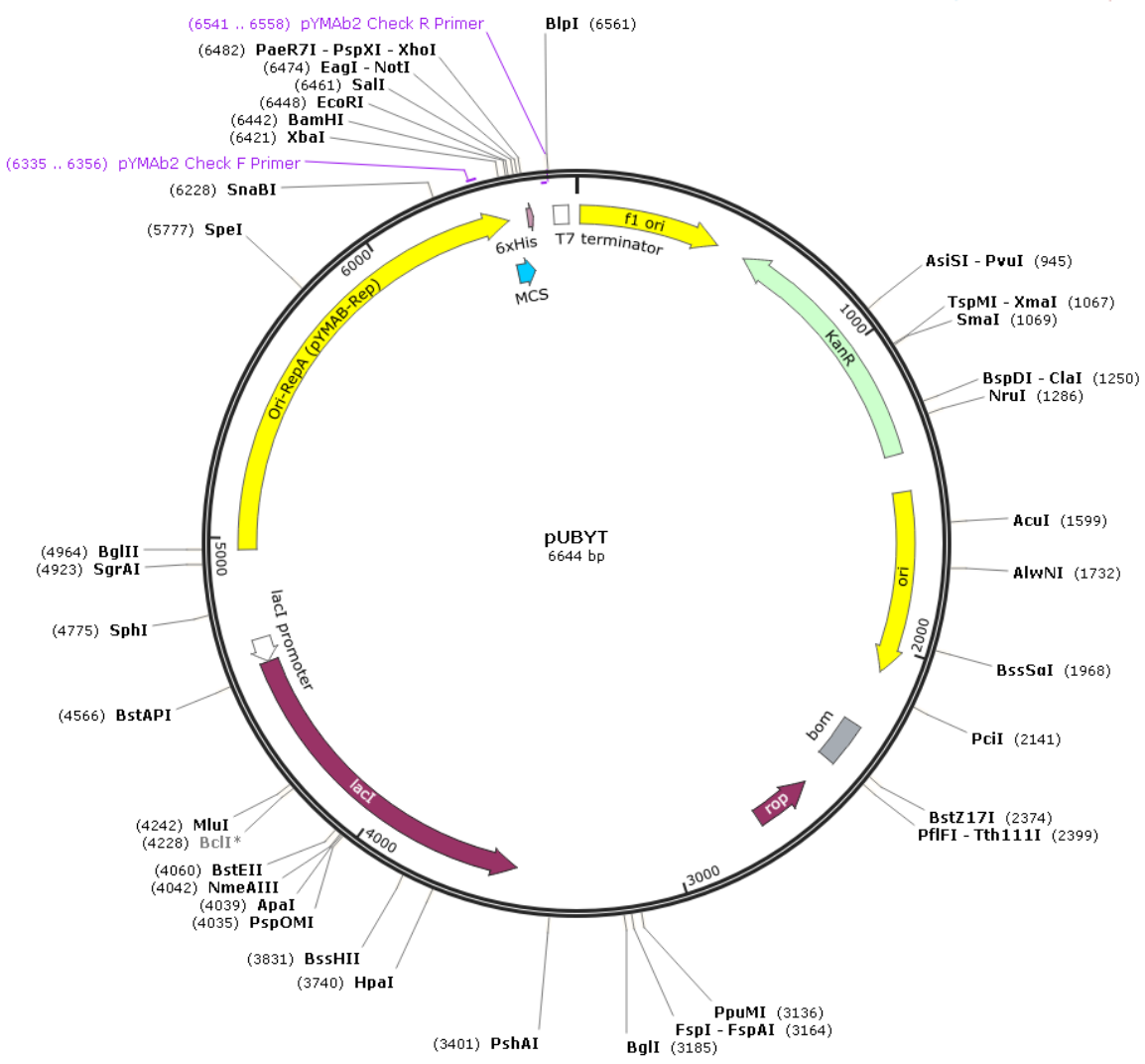

Figure 14. pUBYT vector map. pUBYT vector was designed and made by Dr Yuiko Takebayashi derived from the pYMAb2 plasmid (225). Genes encoding CTX-M-15, KPC-3 and CMY-2 were cloned by Dr Juan-Carlos Jimenez-Castellanos and subsequently sub-cloned into the pUBYT vector by Dr Yuiko Takebayashi (226). KanR is a kanamycin resistance gene and was used to select for successful transformants using 50 mg/L of Kanamycin followed by colony PCR. Figure was created using SnapGene by Dr Yuiko Takebayashi.

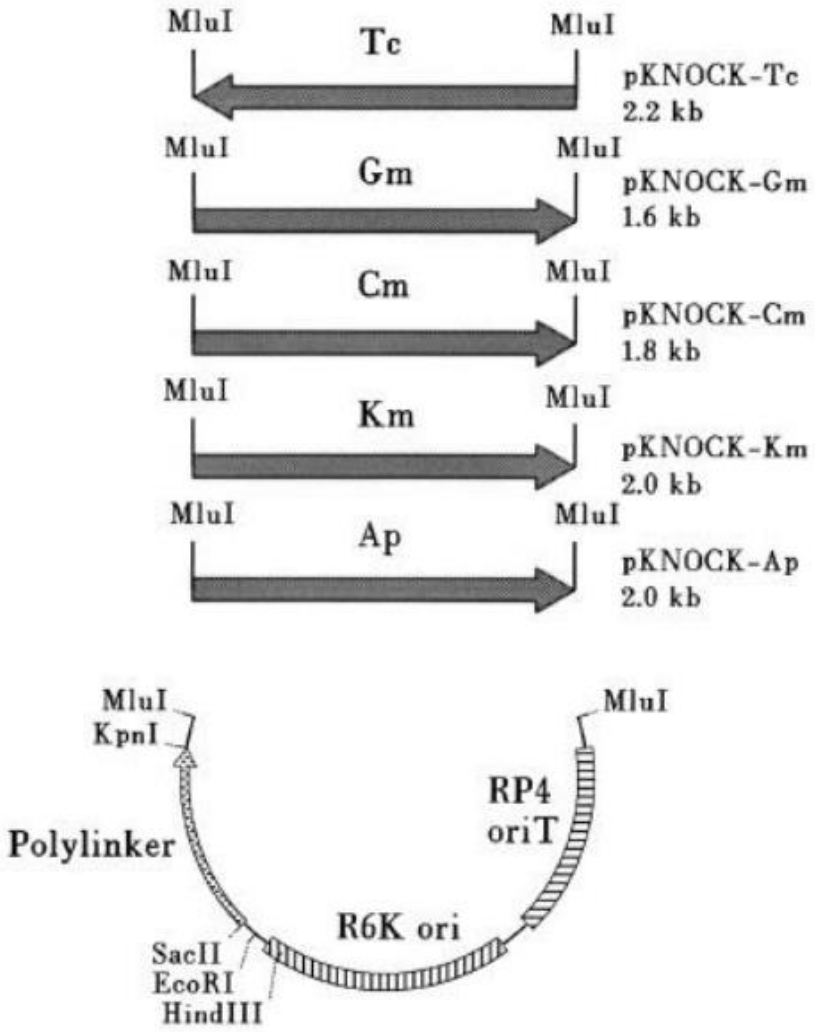

Figure 15. pKNOCK suicide vector map. The pKNOCK vector was kindly gifted by the Chulabhorn Research Institute, Thailand. Knockout primers were used to amplify fragment of desired gene. The gene fragment was then cloned into the pKNOCK vector by visiting PhD student Punyawee Dulyayangkul. The Polylinker in which a gene fragment was inserted, consists of: *EcoR*I-*Sac*II-*Bst*XI-*Not*I-*Eag*I-*Xba*I-*Spe*I-*BamH*I-*Sma*I-*Pst*I-*EcoR*I-*EcoR*V-*Hind*III-*Cla*I-*Sal*I-*Hinc*II-*Acc*I-*Xho*I-*Apa*I-*Dra*II-*Kpn*I. In this case the pKNOCK vector contained the chloramphenicol resistance gene (Cm) or the gentamicin resistance gene resulting in a total size of 1.8 kb pKNOCK-Cm or 1.6 kb pKNOCK-Gm. Figure from (227)

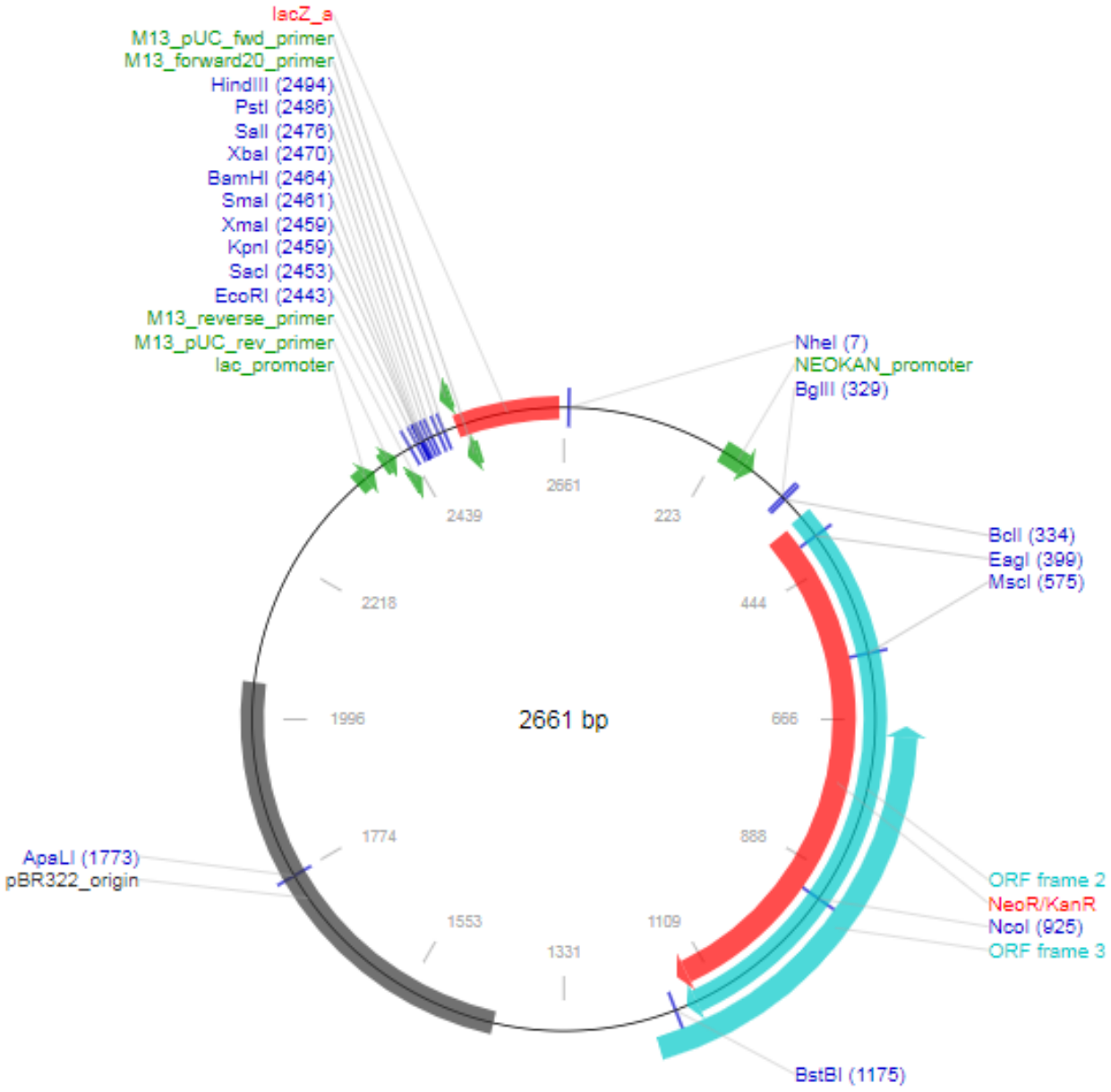

Figure 16. pK18 vector map (228)**.** *micF* was cloned into the pK18 vector by Dr Juan-Carlos Jimenéz-Castellanos (192). NeoR/KanR is a neomycin/ kanamycin resistance gene. In this case 30 mg/L of kanamycin was used to select for successful transformants followed by colony PCR.

2.8. PCR

2.8.1. Crude DNA extraction

DNA was extracted from bacterial cells via boiling on a heating block. One colony was resuspended in 90 µl of sterile Elgastat water. Cells were then boiled at 95^{O}C for 10 min. Cells were subsequently centrifuged at 13,000 rpm for 1 min, whereby 1 µl of the resulting supernatant was used as a template for PCR reactions

2.8.2. Primers

Primers	**Sequence**	**Annealing temperature**
CTX-M 15 F	5'- ACACACGTGGAATTTAGGGA-3'	55.3
CTX-M 15 R	5'- TTTTGCCGTCTAAGGCGATA-3'	55.3
OmpK36 sequencing F	5'- GAGGCATCCGGTTGAAATAG-3'	57.3
OmpK36 sequencing R	5'- ATTAATCGAGGCTCCTCTTAC-3'	55.9
pYT insert check F	5'- GCAAGAAGGTGATGAATCTAC-3'	56.6
pYT insert check R	5'- GTGGCAGCAGCCAACTCA-3'	58.2
OmpK35 F amplification	5'- CACTTCGATGTATTTAACCAG-3'	52.4
OmpK35 R amplification	5'- ATGATGAAGCGCAATATTCTG-3'	54.8
OmpK35 F knockout	5'- TCCCAGACCACAAAAACCCG-3'	59.4
OmpK35 R knockout	5'- CCAGACCGAAGAAGTCGGAG-3'	61.4
OXA 232 check F	5'- GAATGCCAGCGGTAGCAAAG-3'	59.9
OXA 232 check R	5'- CGATATCACGCGTCTGTCCA-3'	59.9
M13 (pK18, pSU18 check) F	5'- GTAAAACGACGGCCAG-3'	52.8
M13 (pK18, pSU18 check) R	5'- CAGGAAACAGCTATGAC-3'	46.6
OmpK36 promoter F	5'- CCAAAGATCAGGGCTTCGGT-3'	59.4
OmpK36 promoter R	5'- ACGACCGTAGTCGAAAGAGC-3'	59.4
NlpD sequencing F	5'-GTCGGCGAAGAGCATCAGT-3'	58.8
NlpD sequencing R	5'-CACCTTCCACGGCACATCA-3'	58.8

Table 7. Primers used, showing oligonucleotide sequence 5'-3' and annealing temperature. OmpK36, OmpK35 and NlpD primers were designed by PhD student Wan Nur

Ismah Kamil. OmpK35 knockout primers were designed by visiting PhD student Punyawee Dulyayangkul. CTX-M 15, OXA 232 and OmpK36 promoter primers were designed personally. All primers were purchased and synthesised by Eurofins Genomics.

2.8.3. PCR amplification

PCR reactions contained 1 µl (10-50 ng) of DNA template, 1 µl (10 ρmol) of forward and reverse primer, 12.5 µl of RedTaq or MyTaq (Sigma-Aldrich) made up to 25 µl by adding 10.5 µl of sterile Elgastat water. PCR reactions were performed in a Bio Rad Peltier Thermal cycler in 0.2 ml thick PCR tubes. The program remained the same in all PCR reactions used, however the annealing temperature differed depending on the primers used (see Table 7). DNA template was denatured at 95^{O}C for 10 min followed by a further 35-37 cycles of denaturing at 95 ^{O}C for 1 min, primer annealing at 50-65^{O}C for 1 min, fragment extension at 72^{O}C for 1 min ending with a final 10 min extension at 72^{O}C before being stored at 4^{O}C. PCR reactions were then analysed by agarose gel electrophoresis

2.8.4. Colony PCR

To confirm successful transformation via electroporation (see section 2.8.), colony PCR was performed. Ten colonies of each transformant were picked and resuspended in PCR master mix (12.5 µl of RedTaq or MyTaq, 1 µl (10 ρmol) of forward and reverse primer (e.g. pYT insert check primer), 10.5 µl of sterile Elgastat water). Colony PCR reactions were then analysed by agarose gel electrophoresis.

2.8.5. Agarose gel electrophoresis

DNA products were run on 1% w/v agarose gels, prepared as follows: 1 g of agarose was dissolved in 100 ml of 1X TAE buffer (0.09 M Tris base, 2 mM Ethylenediaminetetra-acetic acid (EDTA), 0.09 M Boric acid, adjusted to pH 8) followed by addition of Ethidium Bromide to give a final concentration of 0.1 mg/L. Samples were loaded (8 µl) alongside 5 µl of Hyperladder (Bioline, UK) 100 bp/1 kbp depending on product size. Gels were run at 100 V for 30-45 min depending on product size. The resulting gel was then visualised using Syngene G:BOX using the GeneSys software.

2.8.6. PCR purification

When sending PCR products for sequencing, PCR reactions where first confirmed to contain correct product size via agarose gel electrophoresis. Remaining PCR reaction was purified using the QIAquick® PCR purification kit (Qiagen Ltd, UK), where instructions where followed in accordance with the accompanying manufacturers protocol. Purified DNA product was eluted using 30-50 µl of Elution buffer or sterile molecular grade water depending on concentration required. The concentration of the purified DNA product was quantified using a Nanodrop, where sterile molecular grade water was used as a blank for calibration purposes.

2.8.7. DNA sequencing and analysis

Purified DNA (PCR product or Plasmid) was sent to Eurofins genomics for Sanger sequencing. Samples were standardized to a final concentration of 10-50 ng/µl depending on product size, plus 1 µl (10 ρmol) of the relevant forward and reverse primer. Results were analysed using sequence alignment software such as Multialin and Benchling (229, 230).

2.9. Competent cell generation

Electro-competent cells for use in transformation, were generated as follows: 1 colony was inoculated in 10 ml of LB broth (containing antibiotic for selection when needed) and grown overnight in a shaking incubator 180 rpm, 37^{O}C for 20-24 h. 1 ml of overnight culture was sub-cultured in 50 ml of fresh LB broth and grown to OD600 0.4-0.6, in a 250 ml conical flask, 180 rpm, 37^{O}C. Cells were then centrifuged at 4000 rpm, 10 min at 4^{O}C, followed by two washes with ice chilled sterile elgastat water with subsequent centrifugation (4000 rpm, 10 min at 4^{O}C), discarding the supernatant each time. Cells were then resuspended and washed twice with 12% (v/v) glycerol. Aliquots of 100 µl were stored at -80^{O}C for later use for electroporation.

2.10. Plasmid purification

Each bacterium containing a relevant plasmid (pUBYT, CTX-M, KPC, CMY, OXA 232, *micF*, pK18) was grown overnight (180 rpm, 37^{O}C) in LB broth containing 50 mg/L kanamycin (for pUBYT plasmids: CTX-M, KPC, CMY), 30 mg/L kanamycin (for pK18 plasmids: *micF*), 30 mg/L chloramphenicol (for pSU18 plasmids: KPC, CTX-M, CMY) or 8 mg/L piperacillin and 4 mg/L tazobactam for selection of the natural pOXA232 (JX423831) plasmid. Plasmid was then purified using the QIAprep Spin Miniprep Kit (Qiagen Ltd, UK) where instructions where followed in accordance with the accompanying manufacturers protocol. Plasmid was eluted using 30 µl sterile molecular grade water or Elution buffer. Purified plasmid concentration was quantified using a Nanodrop where sterile molecular grade water was used as a blank for calibration purposes. Purified plasmid was stored at -20^{O}C for later use in electroporation

2.11. Electroporation

Electro-competent cells and desired plasmid were thawed on ice. 100 µl of competent cells were mixed with 5 µl plasmid containing desired gene product (pUBYT, CTX-M, KPC or OXA-232). Samples were transferred to an ice chilled 2 mm electroporation cuvette and pulsed using a Bio Rad Gene Pulser at 2.5 kV, 25 µF and 200 Ω. One millilitre of 37^{O}C warmed Super Optimal Broth with Catabolite repression (SOC) was added and immediately transferred to the shaking incubator (180 rpm, 37^{O}C) for 1 h. Cells were then centrifuged at 8000 rpm for 5 min and resuspended in 100 µl of SOC broth. Cells where then spread on selective 1.6% LB agar containing relevant selection. Colony PCR was then performed on resulting transformants to confirm insertion of plasmid.

2.12. Antimicrobial susceptibility testing

2.12.1. Disc susceptibility testing

Antimicrobial disc susceptibility testing was performed to Clinical Laboratory Standards Institute (CLSI) guidelines using Mueller Hinton agar (Sigma-Aldrich) on 15 cm agar plates (231). Bacterial suspensions of OD600 0.1 were made by resuspending bacterial colonies in Phosphate Buffer Saline (PBS) followed by spreading on to the plate. Antibiotic selection and 8 mg/L vaborbactam were added to agar if required. Agar volume was standardised at 50 ml per plate. Plates were incubated in a static incubator at 37^{O}C for 24 h. Assay was

performed in triplicate on all accounts and results reported as the average change in inhibitory zone diameter. Changes of inhibitory zone diameter of ≥2 mm was considered significant based on the previously observed reproducibility of the CLSI disc susceptibility method. Breakpoints of non-susceptibility and susceptibility were determined by the CLSI performance standards (231).

2.12.2. Minimal inhibitory concentration (MIC) assays

MICs were determined according to CLSI microtitre MIC methodology (232). 1:2 dilutions were carried out in a Costar 96-well plate using a starting concentration of 256 mg/L of desired antibiotic. MICs were tested with and without vaborbactam at a final concentration of 8mg/L, which was fixed in each well. Each well contained 180 µl of Cation Adjusted Mueller Hinton broth containing antibiotic and 20 µl of bacterial suspension. Bacterial suspensions of OD600 0.01 were made by resuspending bacterial colonies in Phosphate Buffer Saline (PBS). 20 µl of bacterial suspension was then added to each well giving a final bacterial OD600 of 0.001. Plates were then incubated for 18-20 h at 37^{O}C. Bacterial growth in plates were measured using a POLARstar Omega plate reader at OD600. The MIC was defined as the minimal concentration of antibiotic required to completely prevent bacteria growth determined using the susceptibility/non-susceptibility CLSI performance standards (231). 200 µl of PBS was used as a negative control to indicate zero growth, conversely 180 µl of PBS (without antibiotic) and 20 µl of bacterial suspension to indicate maximum overnight growth.

2.12.3. Chequerboard MIC

Chequerboard MICs (i.e. were two antibiotics were combined) were performed according to CLSI guidelines. 1:2 dilutions of antibiotic ‘A’ (e.g. meropenem) were carried out on the y-axis and 1:2 dilutions of antibiotic ‘B’ (e.g. ceftazidime) were carried out on the x-axis of a Costar 96-well plate. MICs were tested with and without vaborbactam and avibactam at final concentrations of 8mg/L and 10mg/L respectively in each well. Each well contained 180 µl of Cation Adjusted Mueller Hinton broth containing antibiotic ‘A’, antibiotic ‘B’ and 20 µl of bacterial suspension. Bacterial suspensions of OD600 0.01 were made and used as above. Plates were then incubated for 18-20 h at 37^{O}C and read as described above.

2.13. Proteomics

2.13.1 Whole cell protein extraction through Mechanical lysis (Sonication)

Ten millilitres of bacterial overnight culture were inoculated up as previously described in triplicate. The cells were subsequently sub-cultured by adding 1 ml of overnight culture to 50 ml of fresh Cation Adjusted Mueller Hinton broth and grown to mid logarithmic phase (OD600 0.4-0.6) in a 250 ml conical flask. Bacterial cells were then pelleted by centrifugation at 4000 rpm, 15 min, 4^{O}C in a ALC Multispeed Refrigerated Centrifuge. The supernatant was then discarded, and the pellet was resuspended in 30 ml of Tris-hydrochloride buffer (30 mM adjusted to pH 8 with NaOH). Cells were then lysed via sonication, by 1 cm insertion of a 13 mm probe and subjected to 1 s on/ 1 s off pulses for 3 min at 63% amplitude. Cell debris and un-lysed cells where separated from whole cell protein by centrifugation at 8000 rpm for 20 min at 4^{O}C. Whole cell protein samples where then stored at -80^{O}C for later analysis by SDS-PAGE.

2.13.2. Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cell protein samples obtained from sonication were thawed on ice. Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent: 10 µl of protein sample was mixed with 100 µl Bio-Rad Protein Assay Dye Reagent, 890 µl sterile elgastat water and OD595 was subsequently recorded and protein concentration calculated.15 µl of protein sample was added to 15 µl of loading buffer (100 mM Tris adjusted to pH6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue with 20% (v/v) glycerol). The mixture was then heated at 95^{O}C for 5 min. One microgram of protein sample was then loaded onto a 12.5 % SDS-PAGE gel which was made as set out in table 8. The gel was run at 170 V, 300 mA for 10-15 min to give a 1 cm run. The gel was then removed from its mould and stained with InstantBlue (Expedeon ISBIL) for around 10 min until protein bands show up blue. InstantBlue stain was removed and destained using sterile elgastat water. The protein bands were cut out of the gel and analysed as in 2.11.3.

Reagents	Separating Gel (ml)	Stacking Gel (ml)
37:1 30% Bis-acrylamide (161-0158 Bio Rad)	5	2
Separating buffer (1.5 M Tris, 0.4% SDS pH 8.8)	2.5	-
Stacking buffer (0.5 M Tris, 0.4% SDS pH 6.8)	-	2.5
10% Ammonium persulphate	0.1	0.1
TEMED	0.01	0.01
Elgastat water	2.75	4.5

Table 8. Composition of Separating and Stacking gels used for SDS-PAGE. SDS-PAGE was used to test protein presence and abundance. Reagents were mixed, with Ammonium persulphate and TEMED being added just before casting the gel.

2.13.3. Proteomic analysis

Whole cell proteomics was analysed by Dr Kate Heesom in the Proteomics Facility at the University of Bristol. In brief, the 1 cm gel run containing the whole cell protein content was cut out of the gel and digested with trypsin using a ProGest automated digestion unit (Digilab, UK). This resulted in the formation of tryptic peptides which were subsequently fractioned into fragments using a Dionex Ultimate 3000 nanoHPLC system, combined with an LTQ-Orbitrap Velos mass spectrometer (Thermo-Scientic). The resulting proteomics data was analysed using the Proteome Discover software v1.2 (Thermo-Scientic) and compared against the *K. pneumoniae* strain ATCC 700721 on the UniProt MGH 78578 database. Data analysis was carried out using Microsoft excel, whereby a paired t-test was used to test for any significant difference in protein abundance. In this case a p-value of ≤0.05 was considered to be a significant result. The fold change of protein abundance was calculated by averaging the absolute abundances for each of the biological triplicates of the ‘parental’ strain compared to the ‘mutant’ strain. Protein abundances were normalised based on 50S and 30S ribosomal content. This involved averaging the abundances of ribosomal content and dividing all remaining protein abundances by the resulting value.

2.14. Gene Knockout using Bacterial conjugation

Gene knockouts were achieved via the pKNOCK suicide vector method (figure 9). Overnights of a gene knockout donor (e.g. *E. coli* S17 carrying pKNOCK plasmid) and a recipient were set up as previously described in 10 ml of LB broth at 180 rpm, 37^{O}C. The cells were subsequently sub-cultured by adding 1 ml overnight to fresh 20 ml of LB broth. Sub cultures were incubated at 180 rpm, 37^{O}C to mid logarithmic phase OD600 of 0.5 which took roughly 1 h. Bacterial cells were collected by centrifugation at room temperature (RT), 4000 rpm for 10 min using the ALC Multispeed Refrigerated Centrifuge PK 121R. Supernatant was discarded, and bacterial pellet resuspended in 1 ml of fresh LB broth. Mixtures of 1:1, 1:2 and 2:1 (donor (S17): recipient (*K. pneumoniae* Ecl8) ratios were made in 1 ml Eppendorf tubes. 10 µl of bacterial mixtures were added to individual strips of nitrocellulose membrane on 1.6% LB agar. Nitrocellulose membrane strips were left to dry at room temperature followed by overnight incubation at 37^{O}C. Nitrocellulose strips were then placed in 1.5 ml Eppendorf tubes containing 1 ml of LB broth and subjected to vortexing to resuspend bacterial cells. This was followed by spreading on selective media containing pKNOCK selection and 100 mg/L ampicillin plus chloramphenicol or gentamicin used to select for pKNOCK vector. Ampicillin used to kill S17 to ensure any bacteria grown was the recipient *K. pneumoniae* Ecl8 (Amp^{R}) containing the pKNOCK vector. Colony PCR was then performed as described previously, for example using *ompK35* amplification primers against a strain with intact *ompK35*. If colonies of recipient produced a larger PCR product, then the pKNOCK vector had successfully inserted into the chromosome by homologous recombination resulting in knockout of the target gene.

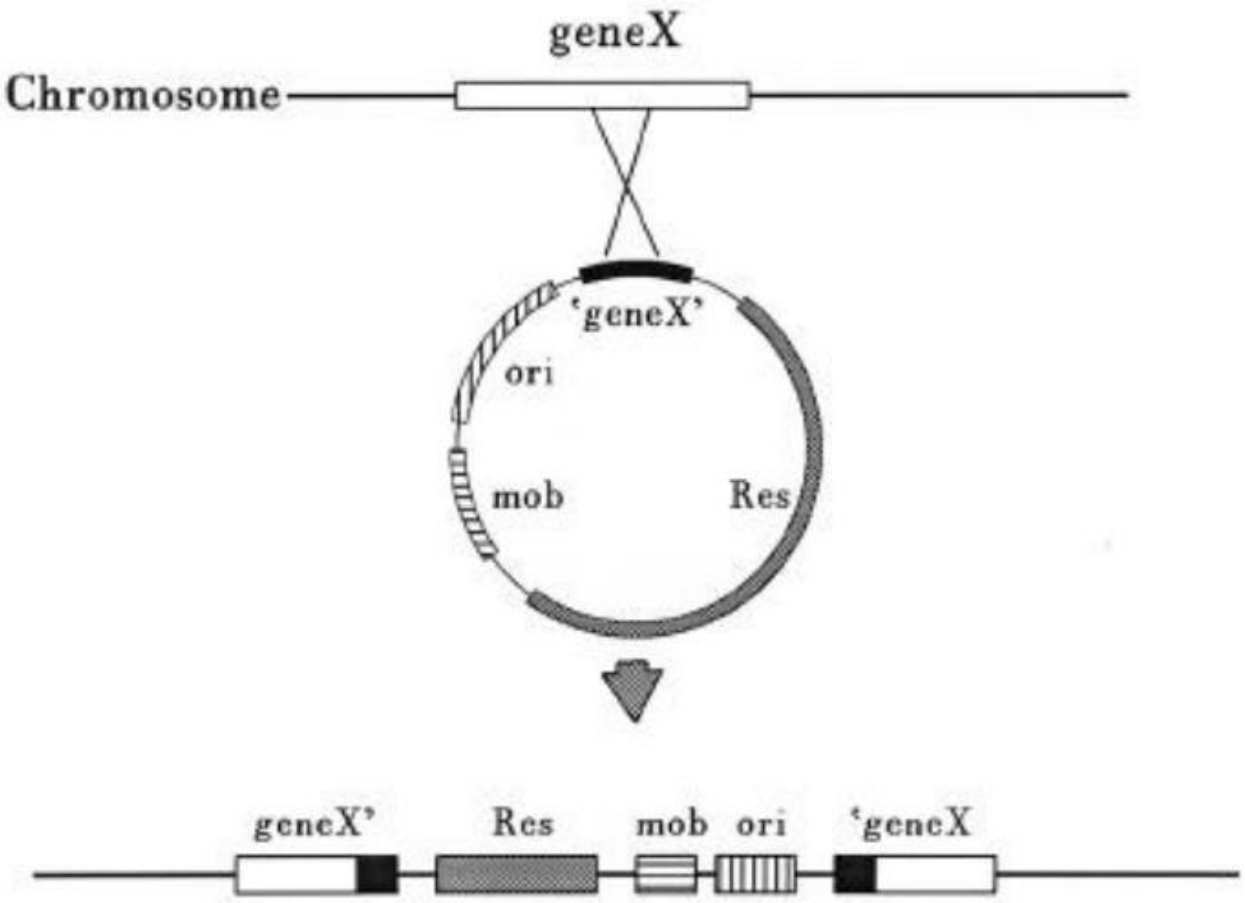

Figure 17. Overview of theory for insertional mutagenesis by pKNOCK suicide vectors. A gene fragment of gene X was cloned into the Polylinker section of the pKNOCK vector. The resulting pKNOCK geneX is mobilised and transported to the recipient by conjugation as described previously. As a result of homologous recombination, the geneX pKNOCK is inserted into geneX on the bacterial chromosome resulting in insertional mutagenesis and knockout of geneX. Figure from (227).

2.15. Selection of β-lactam/β-lactamase inhibitor resistant mutants

Selection of mutants that were resistant to combinations of meropenem/vaborbactam, ceftazidime/vaborbactam and imipenem/vaborbactam was attempted. A range of increasing concentrations of meropenem/ceftazidime/imipenem (0.125 mg/L- 64 mg/L) were tested in the presence of a fixed concentration of vaborbactam (8 mg/L) on Mueller Hinton agar. Overnight bacterial cultures were set up as described previously. 50 µl of overnight culture (grown in Cation Adjusted Mueller Hinton broth) was spread on plates and incubated at 37^{O}C for 24 h. Colonies growing at the highest concentration of meropenem/ceftazidime/Imipenem were selected for further analysis (233).

2.16. Whole genome sequencing

Bacterial strains chosen for whole genome sequencing were grown on Mueller Hinton agar. Glycerol bead stocks were made and sent to MicrobesNG (Birmingham, UK) for genome sequencing on a HiSeq 2500 instrument (Illumina). The reads were subsequently trimmed using Trimmomatic (234) and assembled into contigs by using the SPAdes 3.10.1 software. The resulting data files sent back were: fasta, gbk and trimmed.fastq. These data files were retained for further analysis.

2.17. Whole genome sequence analysis

Three programmes were used for whole genome sequence analysis. These were: Mauve - Multiple Genome Alignment (Mauve 2015-02-26), Galaxy (Galaxy version 18.05) and Intergrative Genomics Viewer-IGV 2.4 (235-237). The assembled contigs for each bacterial strain were mapped to the reference genome of *K. pneumoniae* Ecl8 (GenBank accession number: GCF_000315385.1).

Chapter 3. Factors affecting the ability of vaborbactam to rescue β-lactam resistance.

To test the effect of increased efflux and also porin mutations on the ability of vaborbactam to restore meropenem susceptibility in *K. pneumoniae*, a panel of isogenic strains were created based on the domestic lab strain Ecl8. Plasmids encoding the class A β-lactamase CTX-M or the class D β-lactamase OXA-232 were then used to transform these strains using electroporation as outlined in section 2.8. CTX-M was encoded on the pUBYT vector (see figure 7), whereas OXA-232 was carried on the natural plasmid pOXA232 (JX423831). The efflux and porin mutations present in each Ecl8 derivative is highlighted in table 9. Some of these derivatives were subjected to knockout mutagenesis, as described in 2.14.

Strain	Genotype	Phenotype			
		OmpK35	**OmpK36**	**AcrAB**	**OqxAB**
Ecl8 (WT)	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type
Ecl8 0.03-1 (1)	*oqxR* [c]	Down-regulated ↓	Wild Type	Wild Type	Up-regulated ↑↑
Ecl8 0.03-2 (1)	*ramR* [b]	Down-regulated ↓	Wild Type	Up-regulated ↑	Up-regulated ↑
Ecl8 Delta (1)	Δ*ramR* [a]	Down-regulated ↓	Wild Type	Up-regulated ↑	Up-regulated ↑
Ecl8 35 (1)	Δ*ompK35* [d]	Non-functional	Wild Type	Wild Type	Wild Type
Ecl8 36 (1)	Δ*ompK36* [e]	Wild Type	Non-functional	Wild Type	Wild Type
Ecl8 Delta 35 (2)	Δ*ramR* Δ*ompK35* [d]	Non-functional	Wild Type	Up-regulated ↑	Up-regulated ↑
Ecl8 Delta 36 (2)	Δ*ramR* Δ*ompK36* [e]	Down-regulated ↓	Non-functional	Up-regulated ↑	Up-regulated ↑
Ecl8 44 (2)	Δ*ramR oqxR* [f]	Down-regulated ↓	Wild Type	Up-regulated ↑	Up-regulated ↑↑
Ecl8 Delta 36 micF (3)	*ΔramR ΔompK36* pk18: *micF* [e]	Down-regulated ↓↓	Non-functional	Up-regulated ↑	Up-regulated ↑
Ecl8 16-1 (4)	Δ*ramR oqxR ompK36* [g]	Down-regulated ↓↓	Down-regulated ↓	Up-regulated ↑	Up-regulated ↑↑
Ecl8 0.125 (5)	Δ*ramR oqxR* Δ*ompK36* [h]	Down-regulated ↓↓	Non-functional	Up-regulated ↑	Up-regulated ↑↑

Table 9. Panel of isogenic strains containing efflux and porin modifications. [a] Deletion of *ramR.* [b] *in vitro ramR* point mutant, Thr124Pro. [c] *in vitro oqxR* point mutant Tyr109STOP. [d] *ompK35* knockout, constructed by visiting PhD student Punyawee Dulyayangkul. [e] *ompK36* knockout, constructed by visiting PhD student Punyawee Dulyayangkul. [f] *in vitro* mutant, deletion of *ramR, oqxR* Arg93Cys. [g] *in vitro* mutant, deletion of *ramR, oqxR* Arg93Cys, *nlpD* Asn277FS, *dhaR* Asp395FS (*nlpD* and *dhaR* mutations currently uncharacterised but thought to be linked to porin expression (Kamil et al unpublished)). [h] *in vitro* mutant, deletion of *ramR, oqxR* Arg93Cys, *nlpD* Asn277FS, *dhaR* Asp395FS, *ompK36* Gln170STOP. Arrows indicate the level of down or up-regulation. Ecl8 44, 16.1 and 0.125 mutants selected by Dr Wan Nur Ismah Kamil. Numbers in brackets represent number of mutations present in each strain.

The *K. pneumoniae* lab strain Ecl8 is more permeable than K. *pneumoniae* clinical isolates (238). Accordingly, we found that it was difficult to show increases in β-lactam MIC against these Ecl8 derivatives (in the presence of vaborbactam) as Ecl8 is already highly susceptible, and the MIC assay uses a doubling series scale. The benefit of using antimicrobial disc susceptibility tests is that it provides a continuous scale, and so more clearly showed the different effects that the efflux and porin mutations have on β-lactam/vaborbactam activity. Also, because of this elevated basal permeability for Ecl8, we were less concerned here with whether the efflux or porin mutations gave resistance according to the clinical breakpoints, but more so which mutation or combination of mutations resulted in the largest decrease in inhibition zone diameter when compared to the Ecl8 wild-type strain. Antimicrobial susceptibility testing was carried out in the presence and absence of vaborbactam against a range of β-lactam antibiotics (meropenem MEM, ertapenem ERT, imipenem IPM, doripenem DOR, cefotaxime CTX, cefuroxime CXM, ceftazidime CAZ, ceftriaxone CRO and cefepime FEP). Tables 10-12 show these results, which are summarised below.

Ecl8 0.03-1

This *oqxR* point mutant overproduces the OqxAB efflux pump. Figure 18 indicates, if producing KPC, CTX-M or OXA-232, in both the presence and absence of vaborbactam there is only a limited effect on carbapenem susceptibility when compared to the wild type strain. There was only case of which 0.03-1 produced a significant decrease in zone diameter when compared to Ecl8 WT. Ecl8 WT KPC has a doripenem zone diameter of 31 mm in the presence of vaborbactam, whereas

0.03-1 KPC has a doripenem zone diameter of 25 mm in the presence of vaborbactam. This is a significant decrease *p*=0.0009. Overall, this fits with the recent observation that OqxAB overproduction has little effect on carbapenem susceptibility even in the presence of a β-lactamase (223).

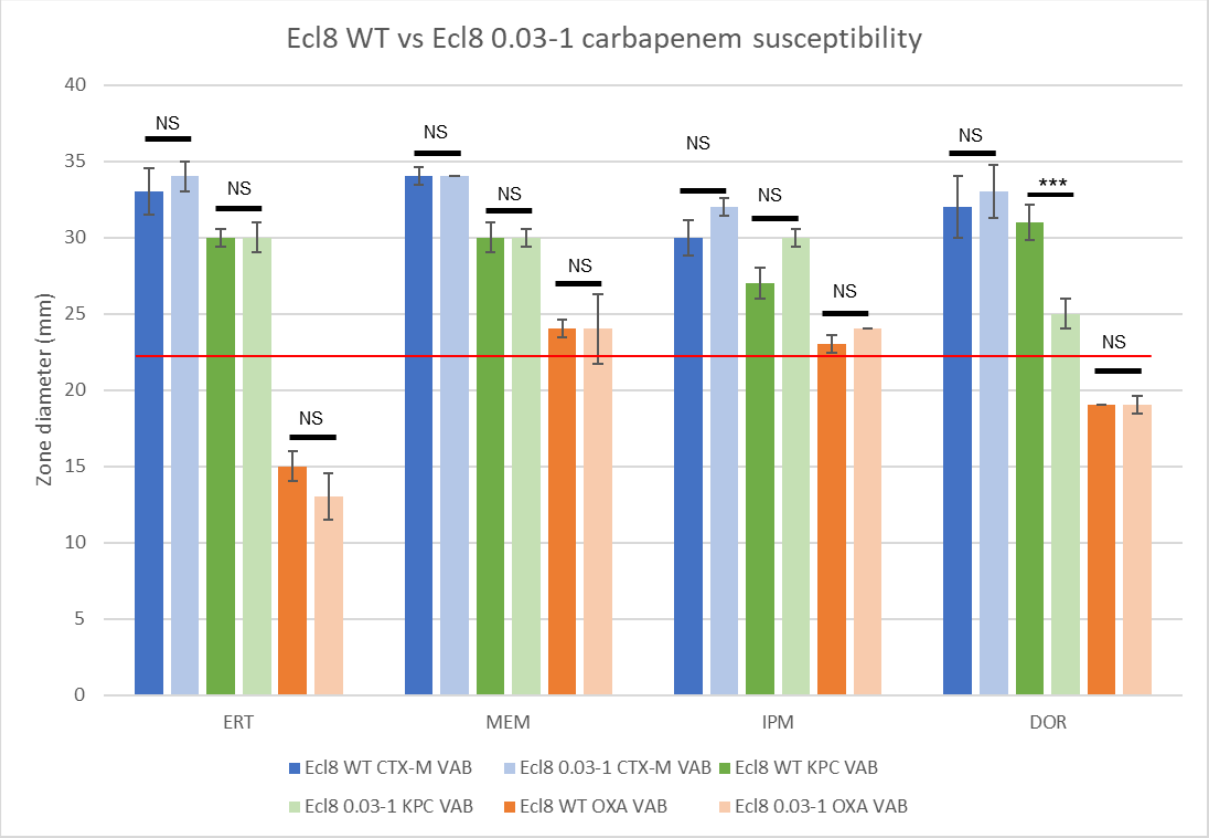

Figure 18. Graphical representation of carbapenem antimicrobial disc susceptibility tests carried out against Ecl8 and Ecl8 0.03-1. The panel of efflux and porin mutants were transformed with plasmids encoding either KPC-3, CTX-M-15 or OXA-232 and tested in the presence and absence of vaborbactam. Disc susceptibility tests were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Data presented is the average of triplicate values. Error bars indicate the variation according to the standard deviation. The black lines indicate significance between Ecl8 0.03-1 and Ecl8 Delta, in this instance 'NS' stands for 'Not Significant, *** = p ≤ 0.001. The red line is the susceptibility cut off for meropenem (i.e. zone diameters above this breakpoint are considered susceptible meropenem).

Ecl8 0.03-2 and Delta

Both are *ramR* mutants, that result in up regulation of the AcrAB and OqxAB efflux pumps, though OqxAB up-regulation is to a lesser extent when compared to *oqxR* mutants (223). Overall RamR loss combined with KPC, CTX-M or OXA-232 production seemed to have a greater effect than OqxR loss on cephalosporin susceptibility. This is probably because AcrAB can efflux cephalosporins more than OqxAB (217) However, like OqxR loss, figure 19 indicates that RamR loss on its own, plays a very minor role in meropenem/vaborbactam susceptibility.

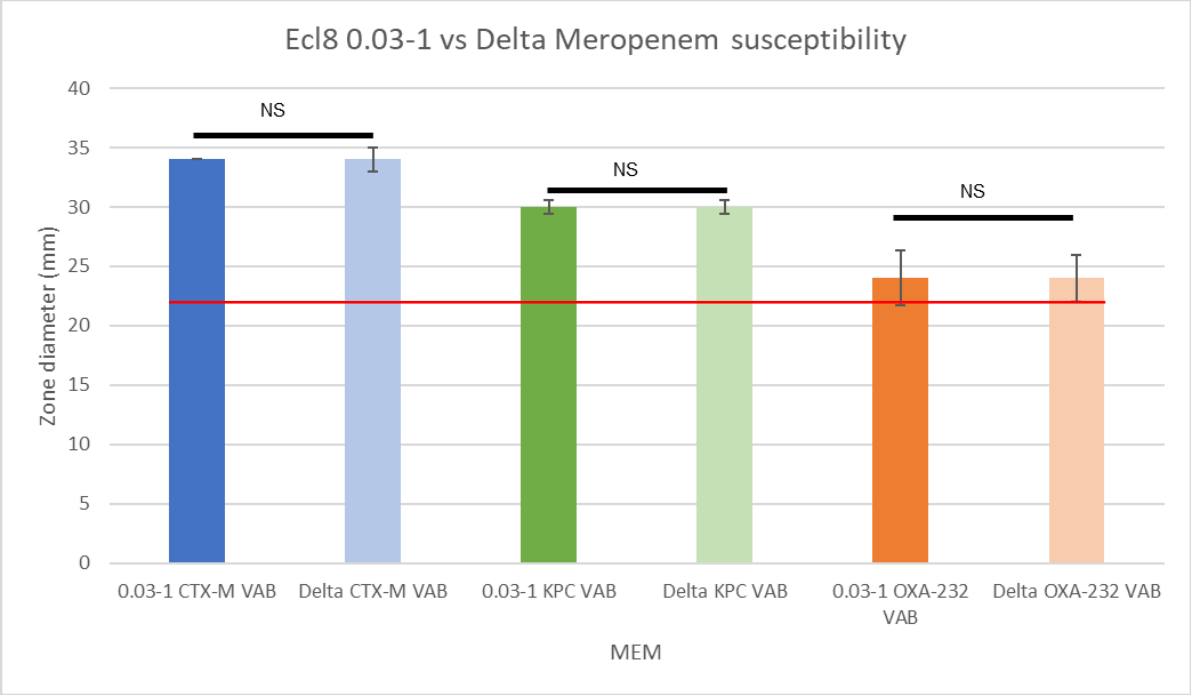

Figure 19. Comparison of meropenem/vaborbactam antimicrobial susceptibility testing between Ecl8 0.03-1 and Ecl8 Delta. The panel of efflux and porin mutants were transformed with plasmids encoding either KPC-3, CTX-M-15 or OXA-232 and tested in the presence and absence of vaborbactam. Disc susceptibility tests were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Data presented is the average of triplicate values. Error bars indicate the variation according to the standard deviation. The black lines indicate significance between Ecl8 0.03-1 and Ecl8 Delta, in this instance 'NS' stands for 'Not Significant. The red line is the susceptibility cut off for meropenem (i.e. zone diameters above this breakpoint are considered susceptible meropenem).

Ecl8 35 and 36

Ecl8 35 and 36 are porin knockout strains of *ompK35* and *ompK36* respectively. The role of porin loss in carbapenem resistance has been well documented, and even implicated in meropenem/vaborbactam non-susceptibility (170, 174, 239). Here we show that loss of OmpK35 results in a decrease in meropenem zone diameter of 4 mm, 0 mm and 1 mm against CTX-M, KPC and OXA-232 respectively, in the presence of vaborbactam, compared to Ecl8 WT (tables 10,11,12). OmpK36 loss had a larger impact with a decrease in meropenem zone diameter, in the presence of vaborbactam, of 6 mm, 2 mm and 2 mm in strains producing CTX-M, KPC and OXA-232 (tables 10,11,12). In fact, figure 20 indicates that the decrease in zone diameter caused by OmpK35 loss in Ecl8 35 KPC and OXA, is not significant. Whereas OmpK36 loss was significant in each transformant. Vaborbactam and meropenem have been reported of having preferential OmpK36 mediated entry (165), so this effect is not surprising.

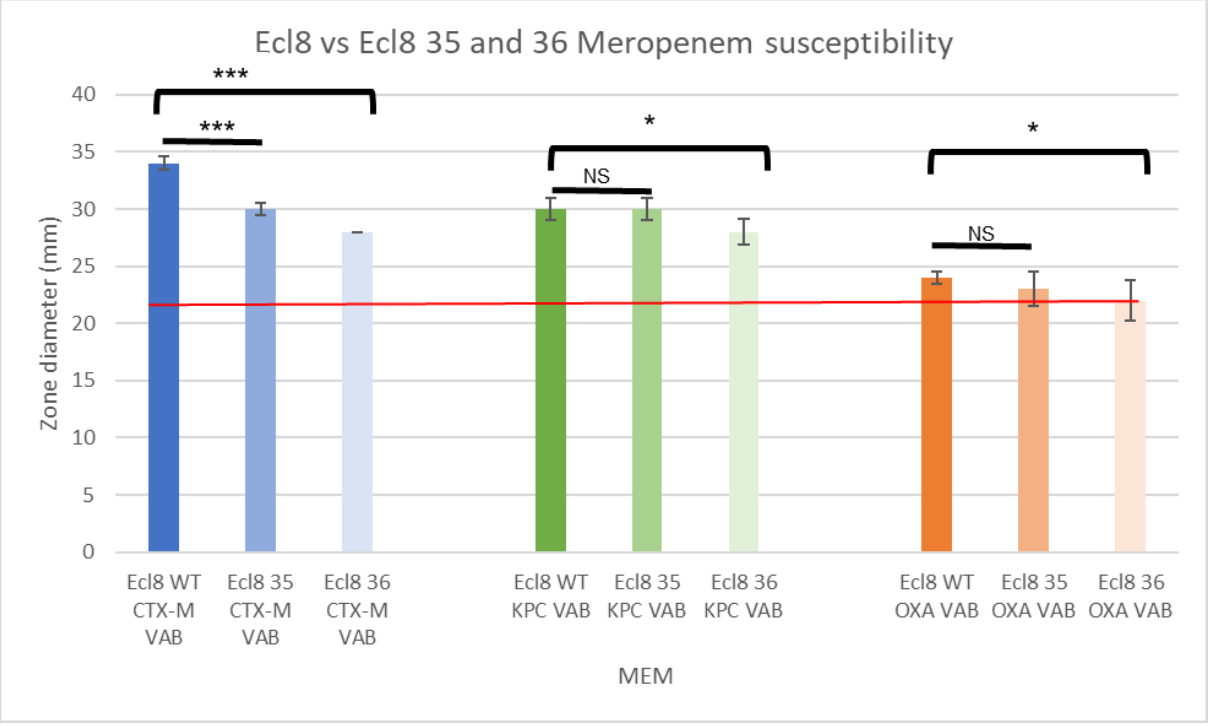

Figure 20. Comparison of meropenem/vaborbactam antimicrobial susceptibility testing between Ecl8 WT, Ecl8 35 and Ecl8 36. The panel of efflux and porin mutants were transformed with plasmids encoding either KPC-3, CTX-M-15 or OXA-232 and tested in the presence and absence of vaborbactam. Disc susceptibility tests were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Data presented is the average of triplicate values. Error bars indicate the variation according to the standard deviation. The black lines indicate significance between Ecl8 0.03-1 and Ecl8 Delta, in this instance 'NS' stands for 'Not Significant, * = $0.01 \leq p \leq 0.05$, *** = $p \leq 0.001$. The red line is the susceptibility cut off for meropenem (i.e. zone diameters above this breakpoint are considered susceptible meropenem)

Ecl8 Delta 35 and Ecl8 Delta 36

Ecl8 Delta 35 and 36 are double mutants that contain a loss of RamR combined with either *ompK35* or *ompK36* knockout. Loss of RamR, which up-regulates AcrAB, on its own was not shown to be a major indicator of meropenem/vaborbactam. However, when combining RamR loss with porin loss, much more significant changes were seen. Figure 21 shows that combining porin loss with efflux over expression resulted in significant decreases in zone diameters for CTX-M, KPC but not for OXA-232. In fact, Ecl8 Delta 36 KPC was meropenem/vaborbactam non-susceptible with a meropenem zone diameter of 21 mm. Therefore, over expression of AcrAB does have an effect on meropenem/vaborbactam susceptibility, even when producing a vaborbactam susceptible carbapenemase, but only when combined with porin loss.

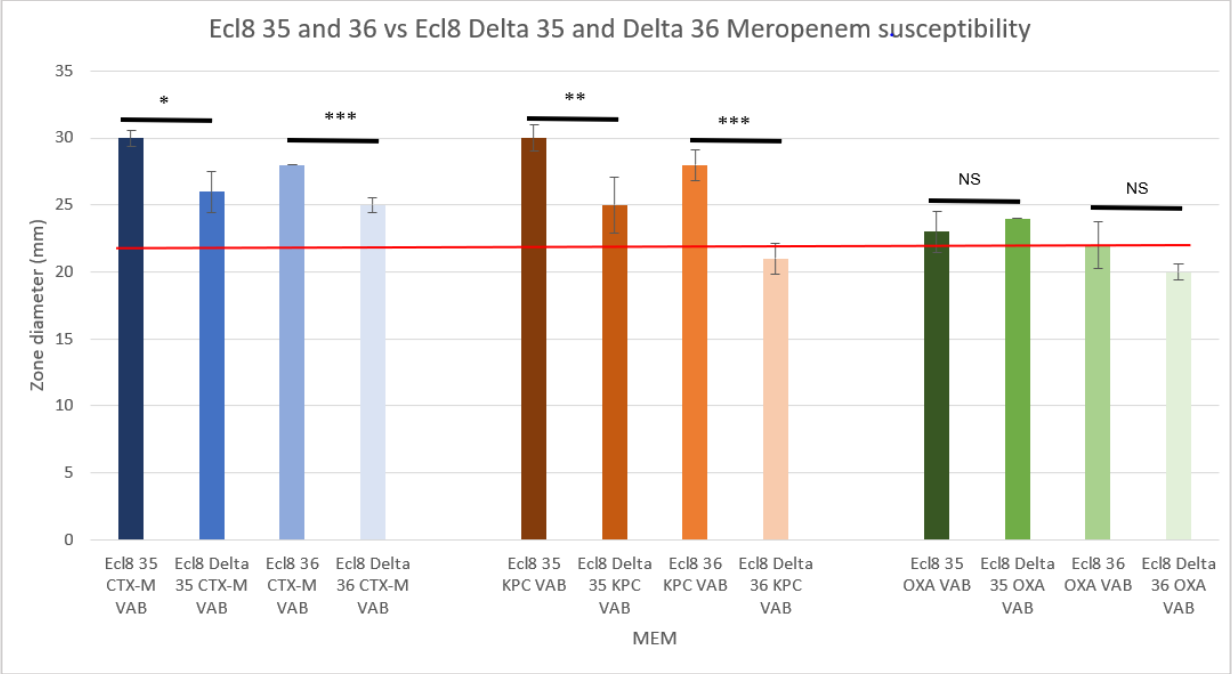

Figure 21 . Comparison of meropenem/vaborbactam antimicrobial susceptibility testing between Ecl8 35, Ecl8 36 and Ecl8 Delta 35 and Ecl8 Delta 36. The panel of efflux and porin mutants were transformed with plasmids encoding either KPC-3, CTX-M-15 or OXA-232 and tested in the presence and absence of vaborbactam. Disc susceptibility tests were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Data presented is the average of triplicate values. Error bars indicate the variation according to the standard deviation. The black lines indicate significance between Ecl8 0.03-1 and Ecl8 Delta, in this instance 'NS' stands for 'Not Significant, * = 0.01 ≤ p ≤ 0.05, ** = 0.001 ≤ p ≤ 0.01, *** = p ≤ 0.001. The red line is the susceptibility cut off for meropenem (i.e. zone diameters above this breakpoint are considered susceptible meropenem).

Ecl8 44

Ecl8 44 is also a double mutant that combines loss of RamR and OqxR. Figure 23 shows that RamR combined with OqxR loss does not lead to a decrease in meropenem zone diameters, in the presence of vaborbactam, against bacteria producing any of the test β-lactamases, compared to Ecl8 0.03-1, 0.03-2 and Delta. This reiterates that efflux, on its own cannot negate the inhibitory capacity of vaborbactam and alter meropenem susceptibility.

Ecl8 Delta 36 micF

This triple mutant combines RamR loss with *ompK36* knockout and OmpK35 loss, achieved through transformation of the inhibitory antisense RNA *micF* on the pK18 plasmid. The loss of OmpK35 further decreases meropenem zone diameters by 3 mm, 1 mm and 2 mm (KPC, CTX-M and OXA-232) in the presence of vaborbactam. However these decreases are only of significance in Ecl8 Delta 36 micF KPC and Ecl8 Delta 36 micF OXA-232 (figure 22). Ecl8 Delta 36 KPC is meropenem/vaborbactam non-susceptible and the over-expression of *micF* further reduced the zone diameter to 18 mm. Additionally, table 11 indicates that Ecl8 Delta 36 mic F KPC was ertapenem, imipenem and doripenem non-susceptible in the presence of vaborbactam.

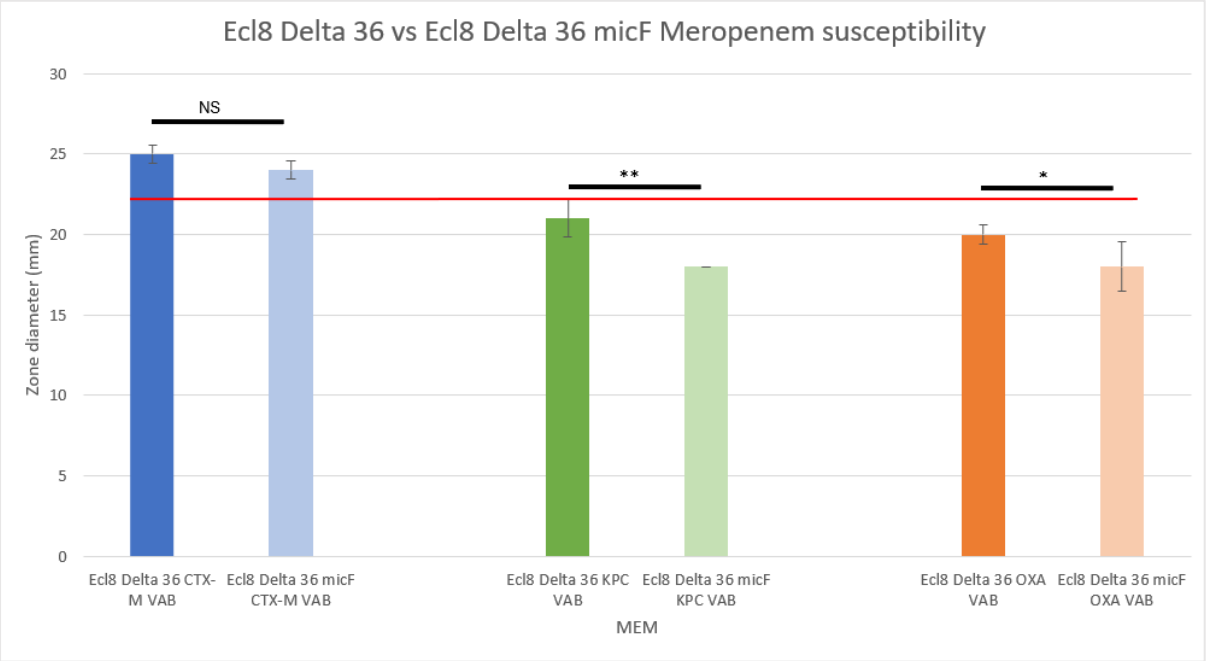

Figure 22. Comparison of meropenem/vaborbactam antimicrobial susceptibility testing between Ecl8 Delta 36 and Ecl8 Delta 36 micF. The panel of efflux and porin mutants were transformed with plasmids encoding either KPC-3, CTX-M-15 or OXA-232 and tested in the presence and absence of vaborbactam. Disc susceptibility tests were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Data presented is the average of triplicate values. Error bars indicate the variation according to the standard deviation. The black lines indicate significance between Ecl8 0.03-1 and Ecl8 Delta, in this instance 'NS' stands for 'Not Significant, * = $0.01 \leq p \leq 0.05$, ** = $0.001 \leq p \leq 0.01$, *** = $p \leq 0.001$. The red line is the susceptibility cut off for meropenem (i.e. zone diameters above this breakpoint are considered susceptible meropenem).

Ecl8 16-1

Ecl8 16-1 is an *in vitro* mutant derived from Ecl8 44. This mutant has the loss of RamR and OqxR that is present in Ecl8 44, as well as mutations in *nlpD*, *dhaR* and down-regulation of OmpK36 (shown by proteomics by Dr Wan Nur Ismah Kamil). However, no mutation was shown to be present in the open reading frame or promoter region of *ompK36.* Neither NlpD or DhaR have previously been implicated in β-lactam resistance. Therefore, one would assume Ecl8 16-1 to have a similar antimicrobial disc susceptibility profile to Ecl8 44. This is clearly not the case as Ecl8 16-1 KPC, CTX-M and OXA-232 has a distinct decreased carbapenem and more so cephalosporin susceptibility both in the presence and absence of vaborbactam. This is highlighted that Ecl8 16-1 and 0.125 OXA are both resistant to cefotaxime (figure 23), even though OXA-232 is not considered a cephalosporinase, Therefore, either NlpD, DhaR or indeed both seem to be a novel cephalosporin resistance mechanism that could potentially be working via reduced porin production.

Ecl8 0.125

Finally, Ecl8 is a mutant which has RamR loss, OqxR loss, *nlpD* and *dhaR* mutations an OmpK36 loss. This OmpK36 loss (combined with all β-lactamases tested) causes a significant decrease in meropenem zone diameter compared to Ecl8 16-1 (figure 23), again highlighting the importance of OmpK36 in meropenem/vaborbactam susceptibility. This mutant has a comparable level of cephalosporin resistance of that shown by Ecl8 Delta 36 micF but shows more susceptibility to carbapenems. Ecl8 0.125 KPC gave meropenem and ertapenem non-susceptibility in the presence of vaborbactam but remained susceptible to doripenem and imipenem. Ecl8 0.125 CTX-M gave ertapenem non-susceptibility in the presence of vaborbactam but remained susceptible to the remaining carbapenems tested. Ecl8 0.125 OXA gave meropenem, ertapenem, imipenem and doripenem non-susceptibility in the presence of vaborbactam.

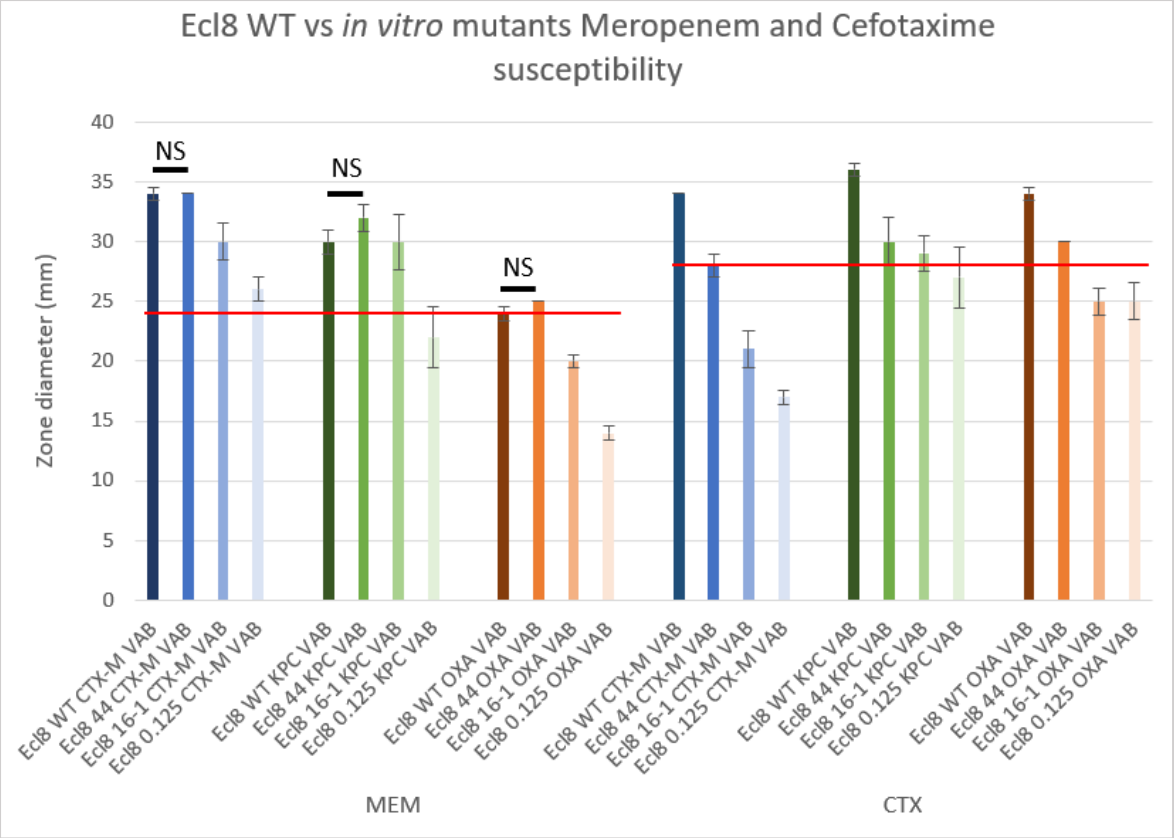

Figure 23 . Comparison of meropenem/vaborbactam antimicrobial susceptibility testing between Ecl8 Delta 36 and Ecl8 Delta 36 micF. The panel of efflux and porin mutants were transformed with plasmids encoding either KPC-3, CTX-M-15 or OXA-232 and tested in the presence and absence of vaborbactam. Disc susceptibility tests were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Data presented is the average of triplicate values. Error bars indicate the variation according to the standard deviation. The black lines indicate significance between Ecl8 0.03-1 and Ecl8 Delta, in this instance 'NS' stands for 'Not Significant' all other decreases in zone diameter compared to Ecl8 WT are in this case of significance ($p \leq 0.05$). The red line is the susceptibility cut off for meropenem and cefotaxime (i.e. zone diameters above this breakpoint are considered susceptible meropenem or cefotaxime).

We have found that of single mutants tested, OmpK36 loss had the greatest impact on meropenem/vaborbactam susceptibility. Although on their own, RamR and OqxR loss did not alter meropenem/vaborbactam activity, when combined with OmpK36 loss resulted in further decrease in meropenem zone diameter in the presence of vaborbactam. Finally, further decrease in meropenem/vaborbactam non-susceptibility can be achieved through mutants that display significant porin and efflux mutations. The most resistant mutants tested contained RamR, OqxR, OmpK36 loss and *nlpD* and *dhaR* mutations (Ecl8 0.125) or RamR, OmpK36 and OmpK35 loss. This is important as it shows a combination of porin loss and increased efflux is required to give high-levels of meropenem/vaborbactam resistance and that many clinical isolates, as shown in chapter 4 already show a significant decrease in permeability.

Additionally, table 11 indicates that vaborbactam is preferentially a KPC inhibitor. Although, still showing a spectrum of activity that includes CTX-M, it is to a lesser extent when compared to vaborbactam inhibition of KPC. When combined with meropenem, this is not so much of a problem, as CTX-M is not a carbapenemase. However, this is highlighted when looking at the antimicrobial disc susceptibility tested of Ecl8 CTX-M against cephalosporins in the presence and absence of CTX-M. Cefotaxime zone diameters of Ecl8 36 CTX-M in the absence of vaborbactam was 6mm, whereas in the presence of vaborbactam, was 23mm, which is non-susceptible according to the CLSI breakpoints. Therefore, when combined with meropenem, the poor carbapenemase capability of CTX-M, rather than vaborbactam inhibitory activity, determines the meropenem/vaborbactam activity. Whereas, when combined with cephalosporins, vaborbactam struggles to restore susceptibility, highlighting the weaker inhibitory capability of vaborbactam against CTX-M. The permeability and efflux modifications also seem to show a larger effect on cephalosporin/vaborbactam susceptibility rather than carbapenem/vaborbactam susceptibility in the presence of CTX-M.

Table 12 shows that vaborbactam is a relatively poor inhibitor of OXA-232. For example, meropenem zone diameter of Ecl8 WT OXA-232 is 26mm, whereas meropenem zone diameter in the presence of vaborbactam is 24mm, showing that vaborbactam is unable to inhibit the carbapenemase potential of OXA-232. Vaborbactam did increase the zone diameters for ertapenem against the OXA-232 mutants, however it was unable to restore susceptibility. The only case of vaborbactam restoring susceptibility was found with Ecl8 44 OXA-232 against cefuroxime (zone diameters of 8 mm and 20 mm respectively). In conclusion, we have found that vaborbactam is a class A β-lactamase inhibitor but shows potency against the KPC carbapenemase. Contrastingly, vaborbactam is a poor inhibitor of OXA-48 like carbapenemases.

	ERT		MEM		IPM		DOR		CXM		CTX		CAZ		FEP		CRO	
		VAB		VAB		VAB		VAB		VAB		VAB		VAB		VAB		VAB
Ecl8 WT CTX-M	27	33 (+6)	30	34 (+4)	28	30 (+2)	28	32 (+4)	6	23 (+17)	11	34 (+23)	23	31 (+8)	21	34 (+13)	11	32 (+21)
Ecl8 0.03-1 CTX-M	26	34 (+8)	30	34 (+4)	30	32 (+2)	30	33 (+3)	6	15 (+9)	6	30 (+14)	15	27 (+12)	16	29 (+13)	6	28 (+22)
Ecl8 0.03-2 CTX-M	25	34 (+9)	30	35 (+5)	29	33 (+4)	29	33 (+4)	6	15 (+9)	6	29 (+23)	11	30 (+19)	15	30 (+15)	6	25 (+19)
Ecl8 Delta CTX-M	25	33 (+8)	31	34 (+3)	29	33 (+4)	29	33 (+4)	6	15 (+9)	6	29 (+23)	11	28 (+17)	14	29 (+15)	6	24 (+18)
Ecl8 35 CTX-M	27	34 (+7)	30	30 (NC)	30	30 (NC)	28	32 (+4)	6	12 (+6)	6	28 (+22)	10	25 (+15)	13	27 (+14)	6	22 (+16)
Ecl8 36 CTX-M	25	29 (+4)	29	28 (+2)	29	29 (NC)	25	25 (NC)	6	10 (+4)	6	23 (+17)	10	27 (+17)	12	25 (+13)	6	23 (+17)
Ecl8 44 CTX-M	15	32 (+17)	30	34 (+4)	28	32 (+4)	25	34 (+9)	6	13 (+7)	6	28 (+22)	14	25 (+11)	12	26 (+14)	6	25 (+19)
Ecl8 Delta 35 CTX-M	25	27 (+2)	30	26 (-4)	29	29 (NC)	26	25 (-1)	6	11 (+5)	6	23 (+17)	9	25 (+16)	12	26 (+14)	6	21 (+15)
Ecl8 Delta 36 CTX-M	24	26 (+2)	28	25 (-3)	28	29 (+1)	20	25 (+5)	6	6 (NC)	6	20 (+14)	7	24 (+17)	10	23 (+13)	6	20 (+14)
Ecl8 Delta 36 micF CTX-M	18	20 (+2)	25	24 (-1)	24	28 (+4)	19	25 (+6)	6	6 (NC)	6	20 (+14)	6	24 (+18)	8	23 (+15)	6	19 (+13)
Ecl8 16-1 CTX-M	15	24 (+9)	26	30 (+4)	25	27 (+2)	21	30 (+9)	6	6 (NC)	6	21 (+15)	7	20 (+13)	8	21 (+13)	6	19 (+13)
Ecl8 0.125 CTX-M	13	20 (+7)	20	26 (+6)	24	25 (+1)	15	26 (+11)	6	6 (NC)	6	17 (+11)	6	19 (+13)	6	16 (+10)	6	15 (+9)

Table 10. Antimicrobial disc susceptibility testing of Ecl8 CTX-M and derived mutants with and without vaborbactam. Green, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation adjusted Ca2+ Mueller Hinton. Assays were performed in triplicate according to CLSI guidelines. Numbers in brackets represent the difference in zone diameter with and without vaborbactam. Changes in zone diameter ≥2 mm is considered a significant change. Data presented as the average zone diameters rounded to the nearest mm.

	ERT		MEM		IPM		DOR		CXM		CTX		CAZ		FEP		CRO	
		VAB		VAB		VAB		VAB		VAB		VAB		VAB		VAB		VAB
Ecl8 WT KPC	15	30 (+15)	20	30 (+10)	19	27 (+8)	21	31 (+10)	15	29 (+14)	24	36 (+12)	24	34 (+10)	26	35 (+9)	23	35 (+12)
Ecl8 0.03-1 KPC	16	30 (+14)	20	30 (+10)	20	30 (+10)	20	25 (+5)	7	22 (+15)	20	34 (+14)	15	30 (+15)	18	29 (+11)	12	30 (+18)
Ecl8 0.03-2 KPC	14	30 (+16)	20	30 (+10)	22	29 (+7)	20	25 (+5)	6	20 (+14)	20	31 (+11)	12	29 (+17)	14	26 (+12)	12	30 (+18)
Ecl8 Delta KPC	14	30 (+16)	20	30 (+10)	21	28 (+7)	19	25 (+6)	6	19 (+13)	20	30 (+10)	11	29 (+18)	12	26 (+14)	12	29 (+17)
Ecl8 35 KPC	6	28 (+22)	14	30 (+16)	16	27 (+11)	15	24 (+9)	6	25 (+19)	8	30 (+22)	6	25 (+19)	6	26 (+20)	6	29 (+23)
Ecl8 36 KPC	6	25 (+19)	12	28 (+16)	13	26 (+13)	11	19 (+8)	6	17 (+11)	6	29 (+13)	6	26 (+20)	6	27 (+21)	6	30 (+24)
Ecl8 44 KPC	15	28 (+13)	21	32 (+11)	21	30 (+9)	22	32 (+10)	6	17 (+11)	22	30 (+8)	17	29 (+12)	19	26 (+7)	19	30 (+11)
Ecl8 Delta 35 KPC	6	18 (+12)	6	25 (+19)	10	25 (+15)	6	15 (+9)	6	14 (+8)	6	30 (+14)	6	22 (+16)	6	22 (+16)	6	30 (+24)
Ecl8 Delta 36 KPC	6	16 (+10)	6	21 (+15)	6	23 (+17)	6	14 (+8)	6	12 (+6)	6	29 (+23)	6	22 (+16)	6	20 (+14)	6	30 (+24)
Ecl8 Delta 36 micF KPC	6	15 (+9)	6	18 (+12)	6	18 (+12)	6	12 (+6)	6	6 (NC)	6	27 (+21)	6	20 (+14)	6	20 (+14)	6	26 (+20)
Ecl8 16-1 KPC	16	24 (+8)	20	30 (+10)	17	30 (+13)	17	32 (+15)	6	14 (+8)	12	29 (+17)	15	28 (+13)	19	21 (+2)	20	28 (+8)
Ecl8 0.125 KPC	6	10 (+4)	6	22 (+16)	6	25 (+19)	6	24 (+18)	6	10 (+4)	10	27 (+17)	8	25 (+17)	7	20 (+13)	6	26 (+20)

Table 11. Antimicrobial disc susceptibility testing of Ecl8 KPC and derived mutants with and without vaborbactam. Green, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation adjusted Ca2+ Mueller Hinton. Assays were performed in triplicate according to CLSI guidelines. Numbers in brackets represent the difference in zone diameter with and without vaborbactam. Changes in zone diameter ≥2 mm is considered a significant change. Data presented as the average zone diameters rounded to the nearest mm.

	ERT		MEM		IPM		DOR		CXM		CTX		CAZ		FEP		CRO	
		VAB		VAB		VAB		VAB		VAB		VAB		VAB		VAB		VAB
Ecl8 WT OXA	15	15 (NC)	26	24 (-2)	27	23 (-4)	24	19 (-5)	25	24 (-1)	31	34 (+3)	35	35 (NC)	35	34 (-1)	33	34 (+1)
Ecl8 0.03-1 OXA	10	13 (+3)	26	24 (-2)	27	24 (-3)	22	19 (-3)	20	19 (-1)	30	30 (NC)	29	26 (-3)	31	28 (-3)	29	27 (-2)
Ecl8 0.03-2 OXA	12	14 (+2)	25	23 (-1)	26	24 (-2)	21	19 (-2)	21	20 (-1)	30	30 (NC)	30	29 (-1)	31	28 (-3)	31	27 (-4)
Ecl8 Delta OXA	11	13 (+2)	25	24 (-1)	26	24 (-2)	21	19 (-2)	21	20 (-1)	30	30 (NC)	29	27 (-2)	31	28 (-3)	31	27 (-4)
Ecl8 35 OXA	12	13 (+1)	24	23 (-1)	25	22 (-3)	20	18 (-2)	22	19 (-3)	30	29 (-1)	30	25 (-5)	31	29 (-2)	30	27 (-3)
Ecl8 36 OXA	13	13 (NC)	23	22 (-1)	23	20 (-3)	19	19 (NC)	20	20 (NC)	30	27 (-3)	29	25 (-4)	29	26 (-3)	28	25 (-3)
Ecl8 44 OXA	13	13 (NC)	24	25 (+1)	24	24 (NC)	20	20 (NC)	8	20 (+12)	29	30 (+1)	28	29 (+1)	32	31 (-1)	29	30 (+1)
Ecl8 Delta 35 OXA	12	12 (NC)	24	24 (NC)	23	20 (-3)	20	19 (-1)	19	20 (+1)	29	27 (-2)	29	36 (+7)	27	35 (+8)	27	36 (+9)
Ecl8 Delta 36 OXA	6	10 (+4)	22	20 (-2)	22	20 (-2)	18	13 (-5)	17	15 (-2)	28	27 (-1)	27	35 (+8)	26	31 (+5)	26	30 (+4)
Ecl8 Delta 36 micF OXA	6	10 (+4)	20	18 (-2)	20	19 (-1)	11	10 (-1)	13	14 (+1)	27	28 (+1)	26	33 (+7)	26	30 (+4)	24	30 (+6)
Ecl8 16-1 OXA	6	6 (NC)	18	20 (+2)	21	22 (+1)	15	15 (NC)	11	11 (NC)	24	25 (+1)	27	27 (NC)	35	30 (-5)	25	27 (+2)
Ecl8 0.125 OXA	6	6 (NC)	11	14 (+3)	12	15 (+3)	7	8 (+1)	12	11 (-1)	25	25 (NC)	28	28 (NC)	26	28 (+2)	25	26 (+1)

Table 12. Antimicrobial disc susceptibility testing of Ecl8 OXA and derived mutants with and without vaborbactam. Green, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation adjusted Ca2+ Mueller Hinton. Assays were performed in triplicate according to CLSI guidelines. Numbers in brackets represent the difference in zone diameter with and without vaborbactam. Changes in zone diameter ≥2 mm is considered a significant change. Data presented as the average zone diameters rounded to the nearest mm.

Chapter 4. Alternative β-lactam/vaborbactam combinations against *K. pneumoniae*

4.1. Vaborbactam is an efficacious inhibitor of class A and C β-lactamases

The spectrum of β-lactamase inhibition provided by vaborbactam was examined using a clinical isolate library, generously gifted by Southmead Hospital, Bristol. The clinical isolate library, outlined in table 13, contained a panel of *K. pneumoniae* strains carrying a diverse range of β-lactamases. These included: the class A β-lactamases KPC-3 (carried on the natural pKpQIL plasmid), CTX-M-15 and various chromosomal TEM and SHV variants, the class D β-lactamases OXA-1, OXA-9 and the OXA-48 like carbapenemase OXA-232, and finally the class B β-lactamases NDM-1 and VIM-1. Additionally, variations of the R21 strain were made by transforming with plasmids encoding CTX-M-15, KPC-3, OXA-232 and the class C enzyme CMY-2.

Isolate	**β-lactamases**	***ramR***	***oqxR***	***ompK35***	***ompK36***
R1	SHV-11	No change	No change	No change	No change
R2	TEM-1, SHV-11, CTX-M-15, OXA-1	*T141I	No change	No change	No change
R3	TEM-1, SHV-28, CTX-M-15, OXA-1	*T141I	No change	Truncated	No change
R4	NDM-1, OXA-232, OXA-1, OXA-9*(2 - stop codon), TEM-1, SHV-28, CTX-M-15	K9I, *T141I	No change	Truncated	No change
R5	SHV-11	*T141I, *194K	oqxR deleted	No change	No change
R6	TEM-1, SHV-83, CTX-M-15, OXA-1	No change	No change	No change	No change
R7	TEM-1, SHV-83, CTX-M-15, OXA-1	No change	No change	No change	No change
R8	TEM-1, SHV-33, CTX-M-15, OXA-1	*T141I	No change	No change	No change
R9	TEM-1, SHV-28, CTX-M-15	Deleted	Truncated at 5' end.	No change	No change

R10	TEM-1, SHV-28, CTX-M-15, OXA-1	*T141I	No change	No change	No change
R11	OXA-232, TEM-1, SHV-1, CTX-M-15	*T141I, M184V, *194K	No change	Truncated	No change
R12	VIM-1, SHV-11	*T141I	D3Y	Truncated	No change
R13	TEM-1, SHV-11, CTX-M-15, OXA-1	Truncated at 3' end.	No change	Truncated.	No change
R14	TEM-1, SHV-11, CTX-M-15, OXA-1	Deleted	No change	L15V	No change
R15	TEM-1, SHV-108, CTX-M-15, OXA-1	No change	No change	No change	No change
R16	TEM-1, SHV-11	*T141I	No change	No change	No change
R17	TEM-1*(del18), SHV-28, CTX-M-15	*T141I	No change	No change	No change
R18	TEM-1, SHV-28, CTX-M-15, OXA-1	A19V, *T141I	No change	No change	No change
R19	TEM-1, SHV-1, CTX-M-15, OXA-1	*T141I, *194K	No change	No change	No change
R20	CTX-M-15, SHV-11	*T141	A19V	No change	No change
R21	TEM-1, SHV-11	F/S at 44.	No change	No change	No change
R21 CTX-M	TEM-1, SHV-11, CTX-M-15	F/S at 44.	No change	No change	No change
R21 KPC	TEM-1, SHV-11, KPC-3	F/S at 44.	No change	No change	No change
R21 OXA	TEM-1, SHV-11, OXA-232	F/S at 44.	No change	No change	No change

R21 CMY	TEM-1, SHV-11, CMY-2	F/S at 44.	No change	No change	No change
R22	TEM-1, SHV-1, CTX-M-15, OXA-1	*T141I, 194K	No change	No change	No change
R23	TEM-1, SHV-1, CTX-M-15, OXA-1	*T141I, *194K	No change	No change	No change
R24	TEM-1, SHV-11, CTX-M-15, OXA-1	No change	No change	L15V	No change
R25	TEM-1, SHV-76, CTX-M-15, OXA-1	*T141I	No change	No change	No change
R26	TEM-1, SHV-11, CTX-M-15, OXA-1	*T141I	No change	L15V	No change
R27	TEM-1, SHV-119, CTX-M-15, OXA-1	*T141I	No change	Truncated	No change
R28	SHV-11	*T141I	Truncated at 3' end.	No change	No change
R29	SHV-11, OXA-1	*T141I	No change	L15V	No change
R30	TEM-1, SHV-11, OXA-9, KPC-3	A40V, *T141I, *194K	V130A	Truncated.	No change

Table 13. *K. pneumoniae* clinical isolate library, expressing a diverse range of β-lactamases. Details of the β-lactamases of each strain are provided, as well as any efflux or porin mutations present (226). *T141I and 194K are changes in amino acid sequence but are not associated with a change in protein function. It is likely that these are due to genetic drift. All mutations were previously characterised by whole genome sequencing (223).

The results of antimicrobial disc susceptibility testing carried out against each of the clinical isolates is shown in table 14. Each isolate was tested against meropenem (disc contained 10 µg) alone and then meropenem in combination with vaborbactam (present in the agar) at a final concentration of 8 mg/L. As expected, strains containing the β-lactamases TEM, SHV and CTX-M were already susceptible to meropenem in the absence of vaborbactam (breakpoint cut of >23mm)

Isolate	**Without vaborbactam**	**With vaborbactam**
R1 (SHV-11)	26.0	28.0 (+2.0)
R2 (TEM-1, SHV-11, CTX-M-15, OXA-1)	27.0	29.0 (+2.0)
R3 (TEM-1, SHV-28, CTX-M-15, OXA-1)	28.0	30.0 (+2.0)
R4 (NDM-1, OXA-232, OXA-1, OXA-9*(2 - stop codon), TEM-1, SHV-28, CTX-M-15)	6.0	6.0 (NC)
R5 (SHV-11)	27.0	30.0 (+3.0)
R6 (TEM-1, SHV-83, CTX-M-15, OXA-1)	29.0	31.0 (+2.0)
R7 (TEM-1, SHV-83, CTX-M-15, OXA-1)	28.0	31.0 (+3.0)
R8 (TEM-1, SHV-33, CTX-M-15, OXA-1)	31.0	32.0 (+1.0)
R9 (TEM-1, SHV-28, CTX-M-15)	29.0	29.0 (NC.0)
R10 (TEM-1, SHV-28, CTX-M-15, OXA-1)	26.0	33.0 (+7.0)
R11 (OXA-232, TEM-1, SHV-1, CTX-M-15)	11.0	15.3 (+4.3)
R12 (VIM-1, SHV-11)	8.0	11.0 (+3.0)
R13 (TEM-1, SHV-11, CTX-M-15, OXA-1)	22.0	27.0 (+5.0)
R14 (TEM-1, SHV-11, CTX-M-15, OXA-1)	29.0	31.0 (+2.0)
R15 (TEM-1, SHV-108, CTX-M-15, OXA-1)	29.0	30.0 (+1.0)
R16 (TEM-1, SHV-11)	30.0	32.0 (+2.0)
R17 (TEM-1*(del18), SHV-28, CTX-M-15)	31.0	33.0 (+2.0)
R18 (TEM-1, SHV-28, CTX-M-15, OXA-1)	30.0	30.0 (NC)

R19 (TEM-1, SHV-1, CTX-M-15, OXA-1)	28.0	31.0 (+3.0)
R20 (CTX-M-15, SHV-11)	25.0	31.0 (+6.0)
R21 (TEM-1, SHV-11)	31.0	31.0 (NC)
R21 CTX-M (TEM-1, SHV-11, CTX-M-15)	31.0	31.0 (NC)
R21 KPC (TEM-1, SHV-11, KPC-3)	6.0	26.0 (+20.0)
R21 OXA (TEM-1, SHV-11, OXA-232)	21.0	21.0 (NC)
R21 CMY (TEM-1, SHV-11, CMY-2)	21.0	27.0 (+6.0)
R22 (TEM-1, SHV-1, CTX-M-15, OXA-1)	30.0	30.0 (NC)
R23 (TEM-1, SHV-1, CTX-M-15, OXA-1)	30.0	30.0 (NC)
R24 (TEM-1, SHV-11, CTX-M-15, OXA-1)	30.0	30.0 (NC)
R25 (TEM-1, SHV-76, CTX-M-15, OXA-1)	30.0	31.0 (+1.0)
R26 (TEM-1, SHV-11, CTX-M-15, OXA-1)	25.0	29.0 (+4.0)
R27 (TEM-1, SHV-119, CTX-M-15, OXA-1)	26.0	28.0 (+2.0)
R28 (SHV-11)	30.0	30.0 (NC)
R29 (SHV-11, OXA-1)	30.0	32.0 (+2.0)
R30 (TEM-1, SHV-11, OXA-9, KPC-3)	6.0	25.0 (+19.0)

Table 14. Antimicrobial disc susceptibility testing results of clinical isolates tested against meropenem and meropenem in combination with vaborbactam. Isolates non-susceptible to meropenem are highlighted in red. Isolates susceptible to meropenem are un-highlighted. Numbers in brackets show the increase in zone diameters. 'NC' denotes an unchanged meropenem zone diameter when combined with vaborbactam (8 mg/L) (present in agar) (226).

Of the 34 clinical isolates tested against meropenem, 26 were susceptible (76.5%). The 8 non-susceptible isolates contained either KPC-3, OXA-232, NDM-1, VIM-1 or CMY-2. It has previously been reported that CMY-2 in combination with a *ramR* mutation gives meropenem non-susceptibility, (192) and isolate R21, which here is producing CMY-2 is a *ramR* mutant. The isolate R13 also showed non-susceptibility to meropenem. We believe this to be due to the presence of a *ramR* mutation as well as a truncated *ompF* and carriage of CTX-M, as all other isolates containing CTX-M-15 (a total of 23) which did not have these permeability defects were susceptible to meropenem (192). We have also already shown that RamR, and OmpK35 loss combined with the expression of CTX-M contributes to meropenem/vaborbactam non-susceptibility in *K. pneumoniae* Ecl8 (figure 18). Indeed, the clinical isolate R14 contains the same panel of β-lactamases as R13 and is a *ramR* mutant, but has an intact ompK35, and is susceptible to meropenem.

When combined with vaborbactam, the number of clinical isolates susceptible to meropenem increased to 30 (88.2%). Vaborbactam was able to restore meropenem susceptibility against R13 (CTX-M-15), R21 (KPC-3), R21 (CMY-2) and R30 (KPC-3). Meropenem susceptibility was recovered, therefore, due to the inhibition of the class A or C enzymes carried in these strains. The greatest increase in zone diameter, when meropenem was combined with vaborbactam, was shown by the two isolates expressing KPC-3. These isolates, R21 KPC and R30, had an increase in zone diameter of 20mm and 19mm respectively. This shows that, in vivo, as well as in vitro, vaborbactam is a class A and C β-lactamase inhibitor but shows a particular and potent inhibitory activity against KPC. A total of 4 strains remained non-susceptible to meropenem even in the presence of vaborbactam. These were R4 (NDM-1, OXA-232, CTX-M-15), R11 (OXA-232, CTX-M-15), R12 (VIM-1) and R21 (OXA-232). It is known that vaborbactam is a poor inhibitor of metallo-β-lactamases and class D OXA-48 like β-lactamases at least in combination with meropenem, so this observation is not a surprise. This agrees with observations found in chapter 3, that vaborbactam is able to efficiently inhibit class A and C enzymes but cannot inhibit class B and D enzymes.

4.2. Meropenem/vaborbactam resistance is associated with a decreased production of the outer membrane porin OmpK36

As vaborbactam is only clinically available with meropenem, mutants of *K. pneumoniae* clinical isolates were selected against meropenem in combination with vaborbactam (see section 2.13.). Ultimately, we wanted to characterise meropenem/vaborbactam resistance. Four strains were chosen for meropenem/vaborbactam resistance selection, these were the

CTX-M-15 producer R13, the KPC-3 producer R30, the OXA-232, CTX-M-15 producer R11 and the OXA-232 transformant R21 OXA. Although R11 and R21 were already resistant to meropenem/vaborbactam, because OXA-232 is not significantly inhibited by vaborbactam, these strains were later used to test alternative partners for use in combination with vaborbactam, therefore mutants exhibiting higher meropenem/vaborbactam MICs derived from R11 (OXA-232, CTX-M-15) and R21 (OXA-232) were also generated.

Strain	**Meropenem (10 µg)**	**Meropenem (10 µg) + vaborbactam (8 mg/L)**
R13 (*ramR, ompK35,* CTX-M-15)	22.0	27.0
R13-1M	20.0 (-2.0)	22.0 (-5.0)
R11 (*ramR, ompK35,* CTX-M-15, OXA-232)	11.0	15.3
R11-64M	6.7 (-3.3)	6.3 (-9.0)
R21 OXA (*ramR,* OXA-232)	21.0	21.0
R21-16M	6.0 (-8.0)	6.0 (-11.0)
R30 (*ramR, ompK35, oqxR,* KPC-3)	6.0	25.0
R30-2M	6.0 (NC)	14.0 (-11.0)

Table 15. Antimicrobial disc susceptibility testing of clinical isolates and derived mutants against meropenem and meropenem/vaborbactam. White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent. Breakpoints are based on a dosage regimen of 1g every 8h.

Table 15 shows the antimicrobial disc susceptibility results of R13 and its meropenem/vaborbactam resistant mutant 1M (where '1M' denotes the concentration of meropenem selected on 1µg/ml), R11 and its meropenem/vaborbactam higher MIC mutant 64M, R21 OXA and its meropenem/vaborbactam higher MIC mutant 16M and R30 and its meropenem/vaborbactam resistant mutant 2M. Confirming the vaborbactam/meropenem resistant phenotypes. Meropenem MICs were also determined against these four clinical isolates and their derived mutants in the presence and absence of vaborbactam. The MIC values are shown in table 16. The result shows a general concordance with the antimicrobial disc susceptibility testing. While R13 is susceptible to meropenem/vaborbactam with an MIC of 0.5 µg/ml, its mutant 1M is non-susceptible with an MIC of 2 µg/ml. Vaborbactam was able to restore meropenem susceptibility in R30 with a decrease in MIC of 256 µg/ml to 1 µg/ml. Whereas vaborbactam was unable to restore sensitivity in the mutant 2M. Meropenem MICs in the presence and absence of vaborbactam remained the same in both R11 and 64M, reiterating the inability of vaborbactam to potentiate the activity of meropenem against OXA-232.

	Meropenem mg/L	
Strain	**—**	**VAB**
R13	2	0.5
1M	4	2
R11	32	32
64M	64	64
R21 OXA	32	32
16M	64	64
R30	256	1
2M	>256	64

Table 16. MICs of clinical isolates and derived mutants tested against meropenem and meropenem/vaborbactam. White, susceptible; Red, non-susceptible. MICs were performed according to CLSI guidelines. Breakpoints are based on a dosage regimen of 1g every 8h.To better understand the underlying meropenem/vaborbactam resistance mechanism, whole cell proteomics was carried out on the four clinical isolates R11, R13, R21 OXA and R30 and compared to their derived mutants 64M, 1M, 16M and 2M, according to the method outlined in section 2.10.3.

4.2.1. Whole cell proteomics

A comparison of the proteomics results of 1M, 64M,16M and 2M was performed to see what proteins were down-regulated or up-regulated in all four relative to their respective parent strains, and therefore whether there were any conserved proteins implicated in meropenem/vaborbactam resistance. Table 21 shows the proteins common to all four mutants that show either up-regulation or down-regulation. As we can see, there is only one protein that is common to all four mutants. This is the outer membrane porin OmpK36. This strongly implicates the importance of *ompK36* loss or downregulation as an indicator of meropenem/vaborbactam non-susceptibility. Interestingly, the mutants 1M and 2M had very similar fold changes of OmpK36, 0.26 and 0.24 respectively (roughly 4-fold down-regulation). Whereas the OXA-232, CTX-M-15 mutant 64M has a fold change of 0.02, which is a 50-fold down-regulation of OmpK36. The OXA-232 mutant 16M has a similar fold change of OmpK36 as seen with 64M, with a 20-fold down-regulation. This could indicate some similarity in the down-regulation mechanism shown in 1M and 2M and perhaps an alternative mechanism in 64M and 16M. It is important to note that all of these clinical isolates are already significantly impermeable with a non-functional *ramR, ompK35* and in some cases *oqxR* mutations. Therefore, it may be that these mutations are required in combination with *ompK36* loss to give meropenem/vaborbactam resistance and that without existing permeability mutations, loss of *ompK36* is not sufficient to give meropenem/vaborbactam non-susceptibility, as suggested in Chapter 3. This requires significant further investigation, perhaps selected for meropenem/vaborbactam resistant mutants in strains that don't have *ramR, oqxR* or *ompk35* mutations or knockout of *ompK36* in an otherwise wild-type clinical isolate background. See section 4.2.5 for whole genome sequencing results of these mutants.

Accession	Description	Fold-change	T-test
Q6SJ71	AAC(6')-Ib	2.48	0.012
L0BW80	Aminoglycoside 6'-N-acetyltransferase	>20	<0.001
A6TC15	Chorismate synthase	>20	<0.001
A6TDK3	Fimbrial like protein	>20	<0.001
A6TDK6	Fimbrial like protein	17.82	0.002
A6TDK4	Fimbrial usher protein	>20	<0.001
E2QPN9	GltX	>20	<0.001
A6TI00	Lipoate protein ligase	>20	<0.001
A6T6J2	Molybdate transport protein	>20	<0.001
A6T6Y8	Outer membrane lipoprotein carrier	2.27	0.021
A6TGR3	Purine biosynthesis protein	>20	<0.001
A6T6P5	Putative ATP dependent RNA helicase	>20	<0.001
A6TGF3	Putative transcriptional regulator	2.03	0.047
A0A0E3GJ34	Uncharacterised protein	>20	<0.001
A6T8F5	Uncharacterised protein	>20	<0.001
A6TCF0	Uncharacterised protein	>20	<0.001
A6TEJ9	Uncharacterised protein	>20	<0.001
A6TGL4	Uncharacterised protein	>20	<0.001
A6TDR7	Aminomethyltransferase	0.42	0.002
A6TGU5	ATP-binding component of transport system for maltose	0.43	0.012
A6TG34	Bifunctional protein	0.49	0.028
A6T7B0	c ycdO	0.41	<0.001
A6THT8	Carbon starvation protein	0.46	0.006
A6TDS4	D-3-phosphoglycerate dehydrogenase	0.47	<0.001
A6T5A4	D-alanine-D-alanine ligase	0.48	0.009
A6TC01	Dihydrofolate: folylpolyglutamate synthetase	0.42	0.004
A6TH73	Fumarate reductase	0.27	0.001
A6T6C1	Glucosamine deaminase	<0.05	<0.001
A6T791	Glucose-1-phosphatase	0.33	0.003
A6TCV4	Glutamate-cysteine ligase	0.49	0.015
A0A0A2RBF5	Glyceraldehyde dehydrogenase	<0.05	<0.001
A6T4P6	GMP reductase	0.49	0.019
A6TD70	L-serine deaminase	0.45	0.001
A6T5S9	Lysine decarboxylase	0.2	<0.001
A6TGU6	Maltoporin 2 LamB	0.31	0.001
A6TBT2	OmpK36	0.26	0.023
A6TG75	Phosphatase	<0.05	<0.001
A6TG32	Phosphate-binding protein	0.37	0.032
A6TBJ7	Phosphomethylpyrimidine kinase	<0.05	<0.001
A6TGQ1	Phosphomethylpyrimidine synthase	0.21	0.006
A6TC99	Phosphoribosylaminoimidazole-succinocarboxamide synthase	0.42	0.047
A6TCB3	Phosphoribosylformylglycinamidine cyclo-ligase	0.47	0.012
W1H7Z5	Phosphoribosylglycinamide formyltransferase	<0.05	<0.001
A6TD69	Probable serine transporter	0.45	0.005
A6TFP1	Putative alpha helix protein	0.41	0.008

A6TEA9	Putative dihydroxyacetone kinase	0.38	<0.001
A6THI5	Putative enzyme contains P-loop	0.41	<0.001
A6TD68	Putative uncharacterised protein	0.22	<0.001
A6TGC4	Regulator for deo operon	<0.05	<0.001
A6TGP6	Thiamine biosynthesis protein	<0.05	<0.001
A6TGP9	Thiamine biosynthesis protein	0.27	0.004
A6TGQ0	Thiamine-phosphate synthase	0.29	0.002
A6TGP7	Thiazole synthase	0.31	0.019
A0A0K0VLC4	Type I restriction enzyme R protein	0.48	0.041

Table 17. Whole cell proteomics results of R13 compared to the derived mutant 1M. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 1M. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

Accession	Description	Fold-change	T-test
A6T8Q6	Fumarase B	>20	<0.001
A6TF05	Nitrite reductase	2.36	0.001
A6T8K6	N-succinylarginine dihydrolase	>20	<0.001
A6T5D9	Preprotein translocase	>20	<0.001
C3TQB2	Pyruvate dehydrogenase	>20	<0.001
Q6KD45	Aerobactin biosynthesis protein	0.44	0.002
T2K1T3	Aerobactin biosynthesis protein	0.35	<0.001
Q3L7J1	Aerobactin siderophore ferric receptor	0.42	0.026
A6T5I0	ATP-dependent Clp protease proteolytic subunit	0.49	0.030
A6TGK6	Conserved protein	<0.05	<0.001
A6T4N5	D-alanine-D-alanine ligase	0.40	<0.001
A6T642	Dihydroxybenzoate synthetase	0.46	0.014
A6TG67	DNA polymerase I	0.34	0.027
A6TIP6	DNA-binding protein	0.47	0.010
A6THJ4	Inosose dehydratase	<0.05	<0.001
A6T640	Isochorismate synthase	<0.05	<0.001
A6TC22	Long-chain fatty acid transport protein	<0.05	<0.001
A6T7A5	Multifunctional: proline dehydrogenase	0.49	<0.001
C9E714	OmpK35	0.05	0.000
A6TBT2	OmpK36	0.02	0.001
A6T631	Outer membrane porin	0.42	0.035
A6THD2	Peptide methionine sulfoxide reductase	<0.05	<0.001
A6T4W5	Periplasmic serine protease	0.42	0.015
A0A0A2R5A5	Porin Morganella	<0.05	<0.001
A6TFG2	Putative outer membrane protein	<0.05	<0.001

A6TI10	Right origin-binding protein	0.49	<0.001
A6T817	Transcriptional regulation of aroF	<0.05	<0.001

Table 18. Whole cell proteomics results of R11 compared to the derived mutant 64M. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 64M. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

Accession	Description	Fold-change	T-test
A6TF05	Nitrite reductase	>20	<0.001
A6TB55	Ribose-5-phosphate isomerase	>20	<0.001
A6T748	3-hydroxydecanoyl dehydratase	0.46	0.037
C3TDX2	3-oxoacyl synthase	<0.05	<0.001
A6TCA1	4-hydroxy-tetrahydrodipicolinate synthase	0.35	0.032
A6T4W3	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	0.40	0.020
A6TFX3	Acetolactate synthase	<0.05	<0.001
A6T6W1	Arginine 3rd transport system periplasmic binding protein	0.36	0.044
A6TCJ1	Autonomous glycyl radical cofactor	0.27	0.013
A6T5R4	Bifunctional protein	0.31	0.042
A6T715	Chromosome partition protein	<0.05	<0.001
A6TGW3	Class B acid phosphatase	<0.05	<0.001
A6T685	Cold shock protein	0.36	0.048
A6TAZ5	Cold shock protein	0.32	0.019
A6TB57	Cytoplasmic ferritin	0.34	0.020
A6TBS4	Ecotin	0.36	0.050
A6T6C8	Ferric uptake regulator	0.36	0.029
A6T6C9	Flavodoxin	0.41	0.045
A6TFR3	Glutamate transport	0.37	0.035
A6T6Q5	Glutamine ABC transporter periplasmic-binding protein	0.39	0.049
A6TFK3	Glycerol-3-phosphate dehydrogenase	<0.05	<0.001
A6TEK2	GTPase	<0.05	<0.001
A6T6X2	Hydroxylamine reductase	<0.05	<0.001
A6TEM9	Mannonate dehydratase	<0.05	<0.001
A6T4X0	Methionine aminopeptidase	0.30	0.037

A6TEU1	Methionyl-tRNA formyltransferase	<0.05	<0.001
A6TBW4	NADH dehydrogenase	<0.05	<0.001
A6TC39	Nucleoside transport protein	<0.05	<0.001
A6TBT2	OmpK36	<0.05	<0.001
A6TCK3	Outer membrane protein	0.46	0.026
A0A0A6U389	Pantothenate synthetase	<0.05	<0.001
A6T693	Penicillin-binding protein 5	0.49	0.042
A6TEY5	Peptidyl-prolyl cis-trans isomerase	0.37	0.044
A6TFN0	Phosphopantothenoylcysteine synthase	<0.05	<0.001
A6T700	Phosphoserine aminotransferase	0.35	0.050
A6T5D9	Preprotein translocase subunit	0.35	0.038
A6TD69	Probable serine transporter	0.40	0.002
A6TEN0	Putative dehydrogenase	<0.05	<0.001
A6T7R8	Putative phosphoesterase	0.44	0.005
A6TC63	Putative uncharacterized protein	0.38	0.045
A6T681	Putative uncharacterized protein	0.36	0.007
A6T6Z5	Pyruvate formate lyase activating enzyme	<0.05	<0.001
A6TGB5	Response regulator	<0.05	<0.001
A6TCD6	RlmN	<0.05	<0.001
A6TGW7	Single-stranded DNA-binding protein	0.44	0.038
A6TH72	Succinate dehydrogenase	<0.05	<0.001
A6T4M9	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	<0.05	<0.001

Table 19. Whole cell proteomics results of R21 OXA compared to the derived mutant 16M. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 16M. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

Accession	Description	Fold-change	T-test
D5KTT8	Conjugal transfer protein	>20	<0.001
A6T8P0	Transcriptional regulation of aerobic, anaerobic respiration	>20	<0.001
A6TGU0	UPF0597 protein	>20	<0.001
A6T5F3	1-deoxy-D-xylulose-5-phosphate synthase	<0.05	<0.001
A6T642	2,3-dihydro-2,3-dihydroxybenzoate synthetase, isochroismatase	0.15	0.000
A6T641	2,3-dihydroxybenzoate-AMP ligase	<0.05	<0.001
A6TF13	3-dehydroquinate synthase	0.20	0.004
A6TGM1	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	<0.05	<0.001

A6TGZ9	Acetyl-coenzyme A synthetase	0.14	0.001
A6TDU9	Agmatinase	<0.05	<0.001
E2QHG9	Alkyl hydroperoxide reductase	0.17	0.000
A6TBZ8	Amidophosphoribosyltransferase	0.13	0.007
A6T6W1	Arginine 3rd transport system periplasmic binding protein	0.17	0.020
A6THH4	Aspartate carbamoyltransferase	0.16	0.018
A0A0A2R888	ATP synthase subunit	0.19	0.001
A6TG36	ATP synthase subunit beta	0.20	0.002
A6T614	Betaine aldehyde dehydrogenase	<0.05	<0.001
Q2V9Y4	Beta-lactamase	<0.05	<0.001
A6T6E9	Citrate synthase	0.17	0.010
A6T4N5	D-alanine--D-alanine ligase	0.20	0.012
C3SX72	Enolase	<0.05	<0.001
A6TBN5	Galactose transport protein	<0.05	<0.001
C3THM2	GlnH protein	0.16	0.003
A6T7C5	Glucans biosynthesis protein	0.18	0.004
A6T6B0	Glutamate/aspartate periplasmic binding protein	0.18	0.002
A6T6Q5	Glutamine ABC transporter periplasmic-binding protein	0.17	0.003
A6TDR5	Glycine dehydrogenase	0.20	0.008
A6T6L1	Histidine ammonia-lyase	0.15	0.001
C3SZX2	Histidine--tRNA ligase	<0.05	<0.001
A6THJ2	Inositol 2-dehydrogenase	<0.05	<0.001
A6T689	Lipoyl synthase	0.19	0.020
A6TFK0	L-lactate dehydrogenase	<0.05	<0.001
A6TGU6	Maltoporin 2 LamB	0.29	0.034
A6TGJ7	Mg2+ transport, system	<0.05	<0.001
W1AXD8	N5-carboxyaminoimidazole ribonucleotide synthase	0.17	0.010
A6T521	Na(+)-translocating NADH-quinone reductase	0.19	0.003
A0A0A2R7X8	NAD(P) transhydrogenase	<0.05	<0.001
A6TBX0	NADH dehydrogenase	0.20	0.006
A6T751	OmpA	0.32	0.011
A6TDJ4	OmpK26	0.22	0.011
A6TBT2	OmpK36	0.24	0.003
A0A0A2R8C1	Peptide ABC transporter substrate-binding protein	0.17	0.005
A6TGU4	Periplasmic maltose-binding protein	0.17	0.014
A6T821	Periplasmic murein tripeptide	<0.05	<0.001
A6TF26	Phosphoenolpyruvate carboxykinase	0.20	0.008
A6TGR2	Phosphoribosylamine--glycine ligase	0.14	0.005
A6TC99	Phosphoribosylaminoimidazole-succinocarboxamide synthase	0.20	0.008

A6TCB3	Phosphoribosylformylglycinamidine cyclo-ligase	0.18	0.007
W1DKE2	Phosphoribosylglycinamide formyltransferase	<0.05	<0.001
W1HLQ4	Phosphoribosylglycinamide formyltransferase	<0.05	<0.001
A0A037YAR3	Phosphoserine aminotransferase	<0.05	<0.001
A6TDP7	Protein disulfide isomerase	<0.05	<0.001
A6TDI6	Putative ABC transporter periplasmic binding protein	0.20	0.005
A6TEL4	Putative ATP-binding component of a transport system	0.20	0.008
A6THT6	Putative synthesis protein	<0.05	<0.001
A6T7H0	Putative TonB-dependent receptor	0.15	0.011
A6T7B0	Putative uncharacterized protein	0.18	0.008
A6T5B0	Pyrroline-5-carboxylate reductase	<0.05	<0.001
A6TGC4	Regulator for deo operon	<0.05	<0.001
A6T5E7	Riboflavin biosynthesis protein	<0.05	<0.001
A6TCI1	Ribonuclease	<0.05	<0.001
A6TGE6	Soluble pyridine nucleotide transhydrogenase	0.20	0.001
A6T7J1	Spermidine/putrescine ABC transporter	0.13	0.000
A6TCM7	SsrA-binding protein	0.08	0.000
A6T6F3	Succinate dehydrogenase	0.20	0.006
A6T4K6	Thiamin-binding periplasmic protein	<0.05	<0.001
A6TDG3	Thymidylate synthase	<0.05	<0.001
A6T7W6	Tryptophan synthase	<0.05	<0.001
A6TBF7	Tyrosine autokinase	0.17	0.001
A6T6L0	Urocanate hydratase	0.15	0.011

Table 20. Whole cell proteomics results of R30 compared to the derived mutant 2M. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 2M. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

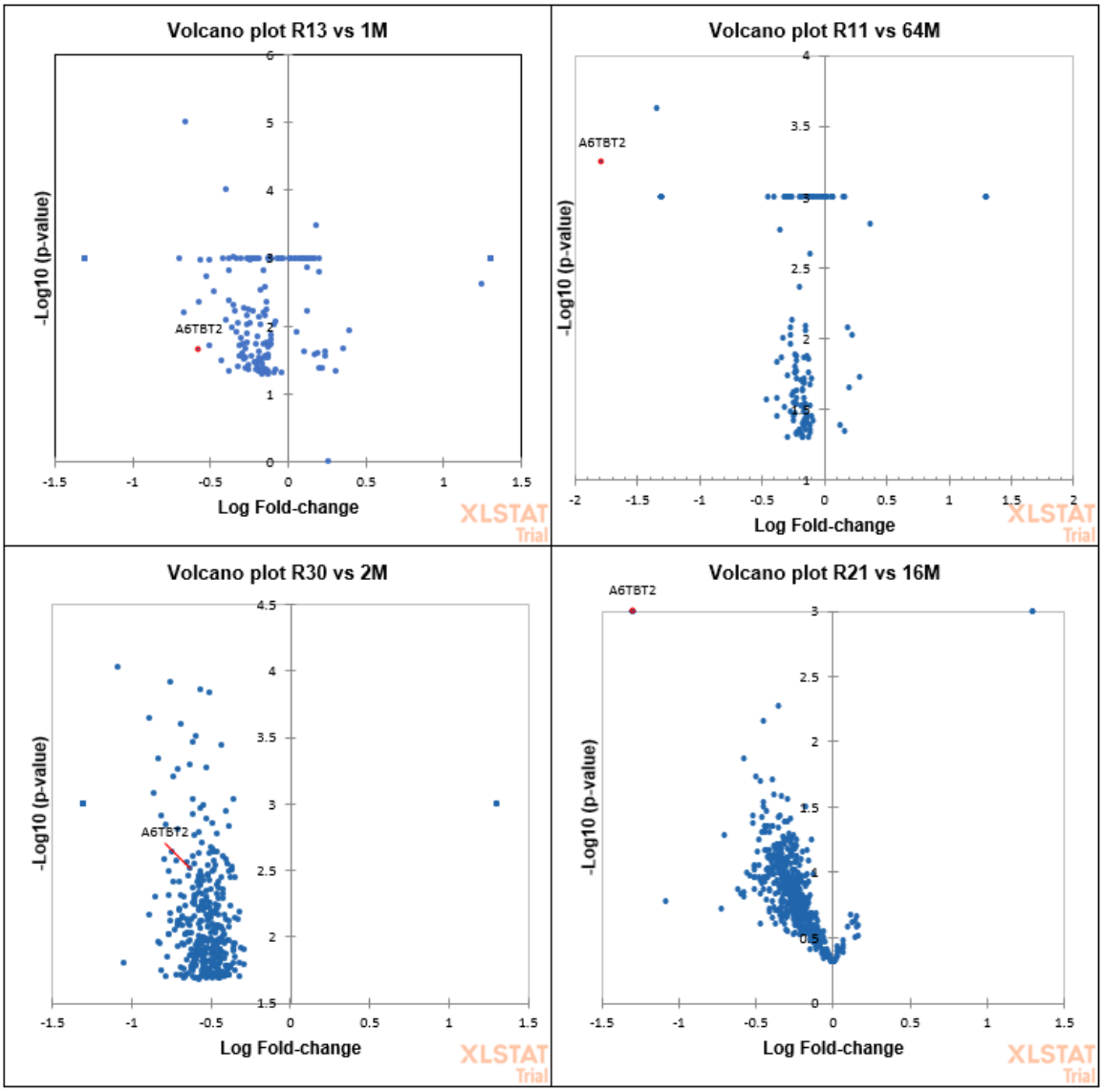

Figure 24. Whole cell proteomics Volcano-plots of clinical isolates vs mutants. Data points < -0.3 x-axis, > 1.3 y axis and > 0.3 x-axis, >1.3 y-axis is considered a significant fold change. OmpK36 is highlighted in red. Figure was made using XLSTAT.

	Accession	Description	Fold-change	T-test
1M	A6TBT2	OmpK36	0.26	0.023
64M	A6TBT2	OmpK36	0.02	0.001
16M	A6TBT2	OmpK36	0.05	0.001
2M	A6TBT2	OmpK36	0.24	0.003

Table 21. A comparison of whole cell proteomics between the four meropenem/vaborbactam resistant mutants 1M, 64M, 16M and 2M. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutants. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

4.2.5. Whole Genome sequence analysis

4.2.5.1. Whole genome sequence indicates insertion sequence in the *ompK36* promoter of 64M, and introduction of a stop codon in 16M

Upon analysis of the whole genome sequence of the meropenem/vaborbactam resistant mutant 64M and alignment to its parent R11 using progressive Mauve (235). A 263 bp insertion sequence was found in the promoter region of *ompK36* in 64M that was absent in the parental clinical isolate R11 (figure 25). The 263 bp insertion sequence was identified as a transposase: protein id AOR89446.1 when using blastn (240). This insertion sequence is likely to be responsible for the decreased OmpK36 production seen in 64M relative to R11 in the whole cell proteomics data. Whole genome sequence alignment using progressive Mauve also indicated an *ompK36* mutation in the mutant 16M. In this case, a stop codon was introduced at position 373 (figure 26) resulting in OmpK36 loss (figure 27, 28). However, no *ompK36* mutation (figure 29, 30) could be found within the meropenem/vaborbactam resistant mutants 1M or 2M having relatively small reductions in OmpK36 production. This means there must be an alternative mutation that can give meropenem/vaborbactam resistance, via downregulation of OmpK36.

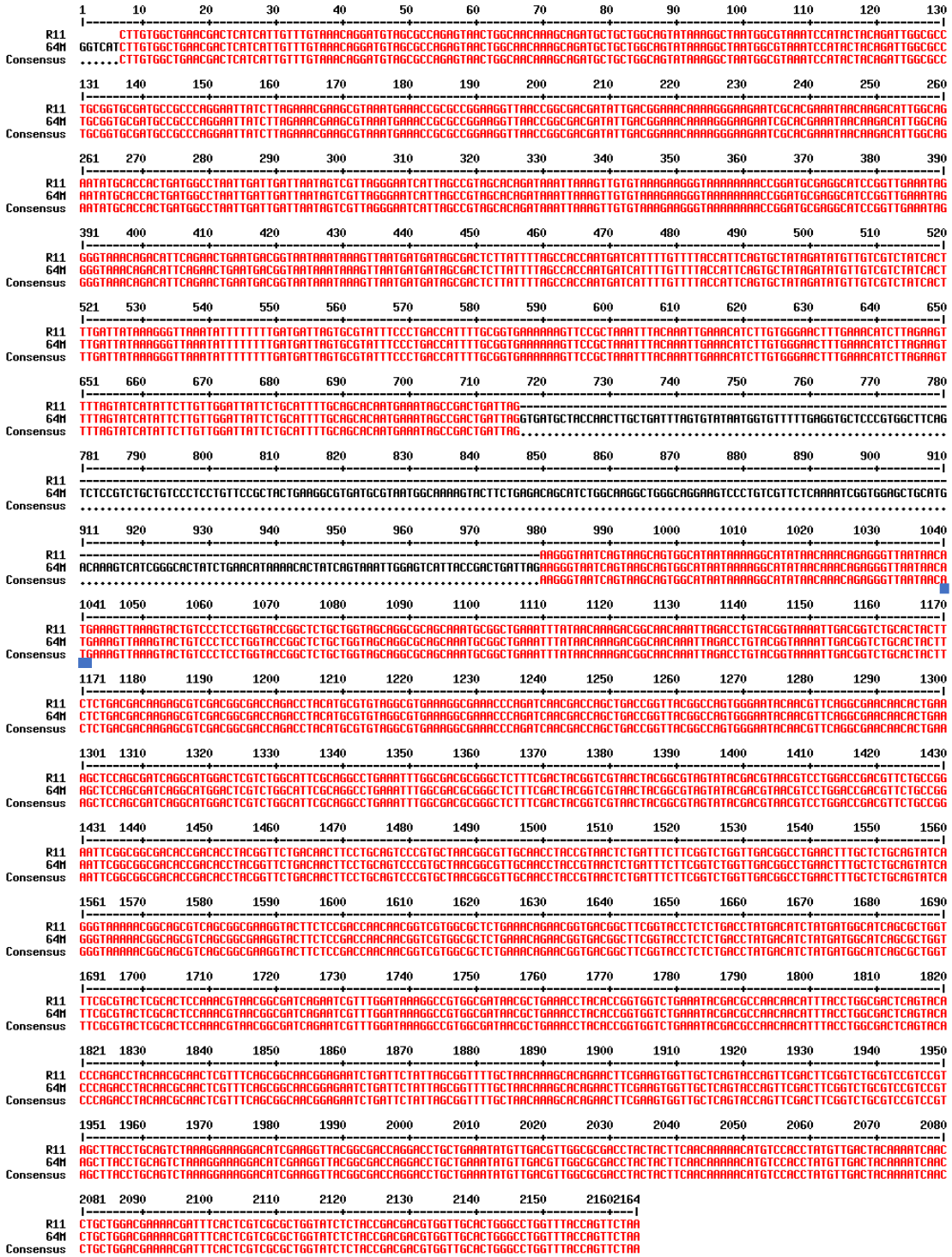

Figure 25. Intergenic and coding regions of *ompK36* in R11 and 64M. Sequence highlighted in red shows consensus between R11 and 64M. Sequence highlighted in black indicates unique sequence not present in R11. *ompK36* start codon is at position 1040 (underlined in blue) with its stop codon at position 2164. The stop codon of the previous gene *rcsD* is at position 58.

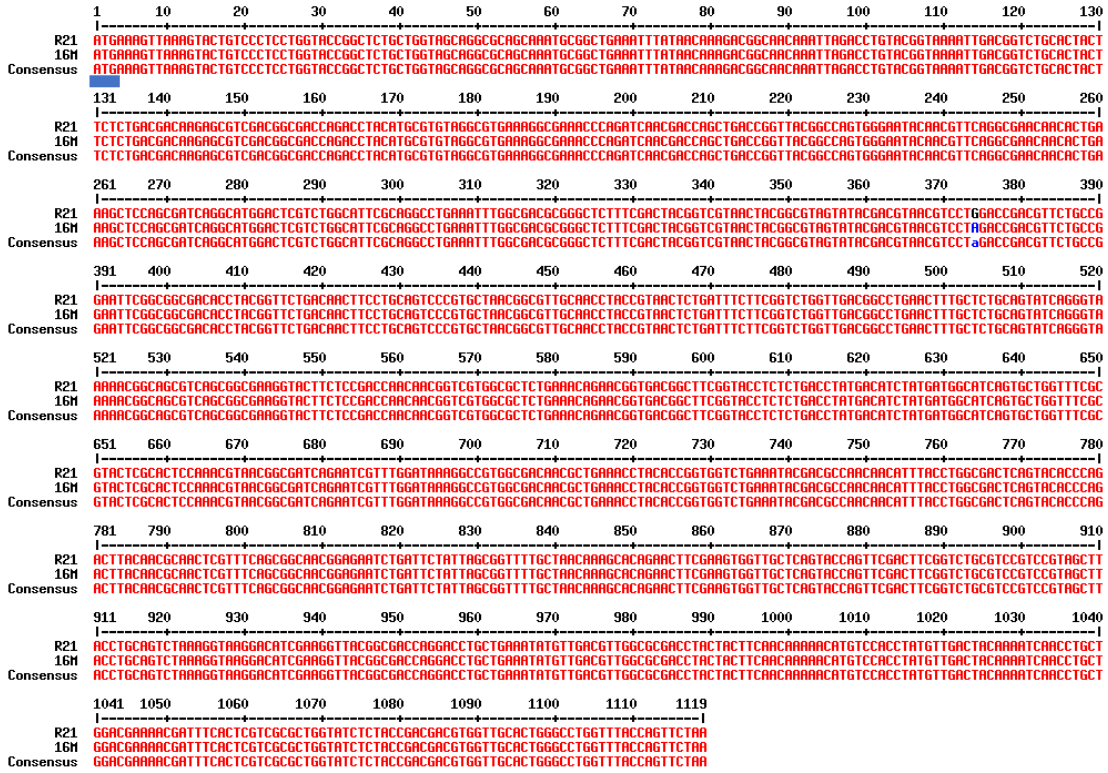

Figure 26. Coding regions of *ompK36* in R21 and 16M. Sequence highlighted in red shows consensus between R21 and 16M. Start codon is at position 1 (underlined in blue). Sequence highlighted in blue indicates a mismatch. In this case a stop codon is introduced at position 373.

```
R21     MKVKVLSLLVPALLVAGAANAAEIYNKDGNKLDLYGKIDGLHYFSDDKSVDGDQTYMRVG   60
16M     MKVKVLSLLVPALLVAGAANAAEIYNKDGNKLDLYGKIDGLHYFSDDKSVDGDQTYMRVG   60
        ************************************************************

R21     VKGETQINDQLTGYGQWEYNVQANNTESSSDQAWTRLAFAGLKFGDAGSFDYGRNYGVVY   120
16M     VKGETQINDQLTGYGQWEYNVQANNTESSSDQAWTRLAFAGLKFGDAGSFDYGRNYGVVY   120
        ************************************************************

R21     DVTSWTDVLPEFGGDTYGSDNFLQSRANGVATYRNSDFFGLVDGLNFALQYQGKNGSVSG   180
16M     DVTS------------------------------------------------------   124
        ****

R21     EGTSPTNNGRGALKQNGDGFGTSLTYDIYDGISAGFAYSHSKRNGDQNRLDKGRGDNAET   240
16M     ------------------------------------------------------------   124

R21     YTGGLKYDANNIYLATQYTQTYNATRFSGNGESDSISGFANKAQNFEVVAQYQFDFGLRP   300
16M     ------------------------------------------------------------   124

R21     SVAYLQSKGKDIEGYGDQDLLKYVDVGATYYFNKNMSTYVDYKINLLDENDFTRRAGIST   360
16M     ------------------------------------------------------------   124

R21     DDVVALGLVYQF   372
16M     ------------   124
```

Figure 27. Protein alignment of OmpK36 between R21 and 16M. '*' indicates sequence consensus between R21 and 16M. **'-'** indicates sequence present in R21 but not present in 16M. Protein sequence was analysed using Clustal Omega (241-243).

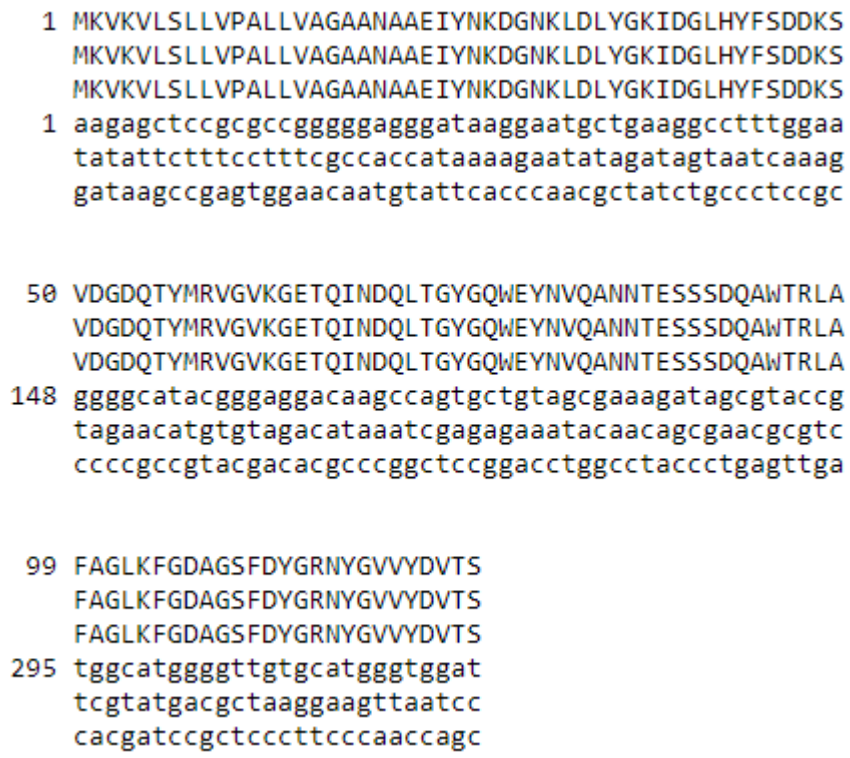

Figure 28. OmpK36 DNA and amino acid sequence pairwise alignment in 16M. Figure was created using EMBL-EBI GeneWise Pairwise Sequence Alignment.

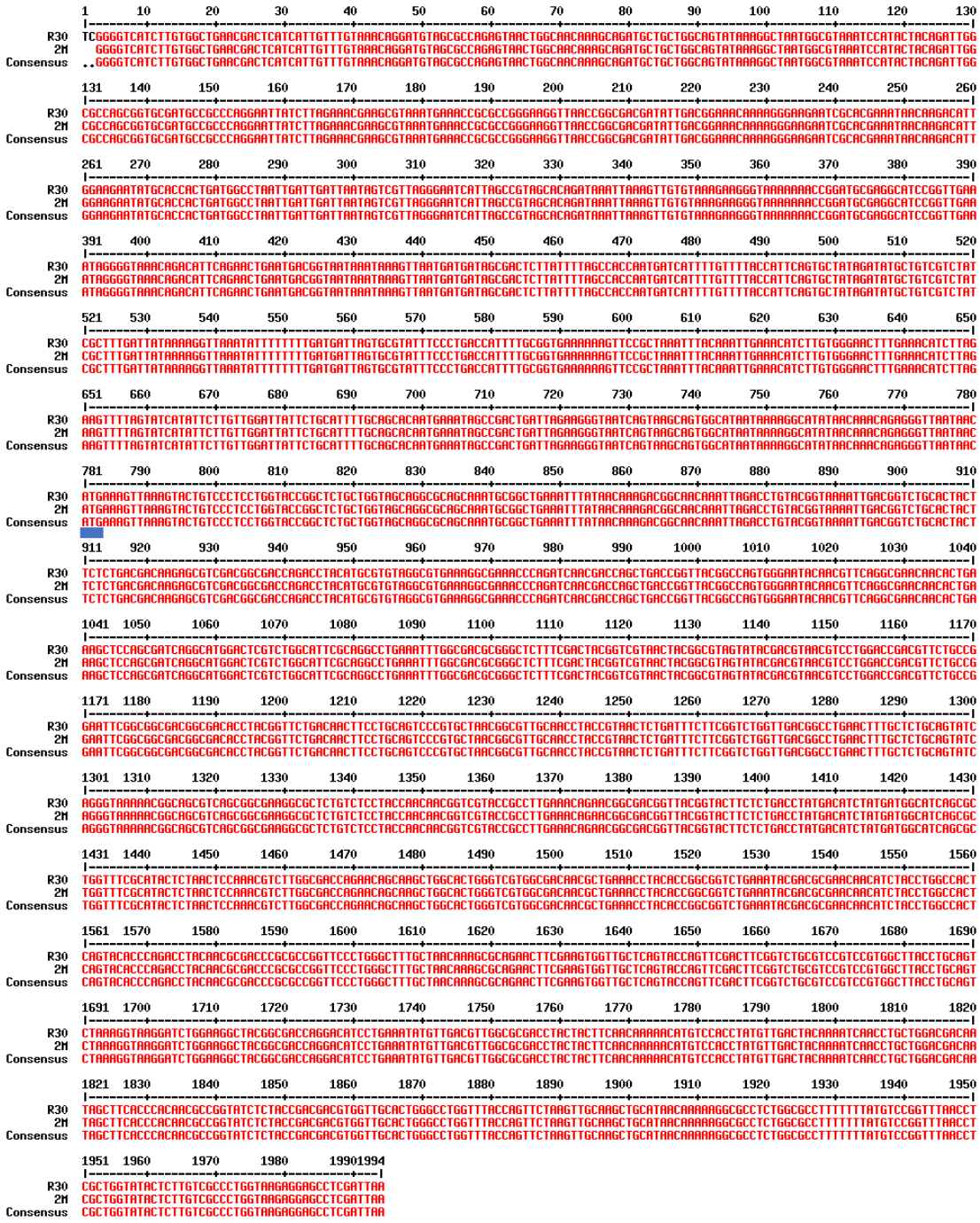

Figure 29. Intergenic and coding regions of *ompK36* in R30 and 2M. Sequence highlighted in red shows consensus between R30 and 2M. *ompK36* start codon is at position 781(underlined in blue) with its stop codon at position 1994. The stop codon of the previous gene *rcsD* is at position 177

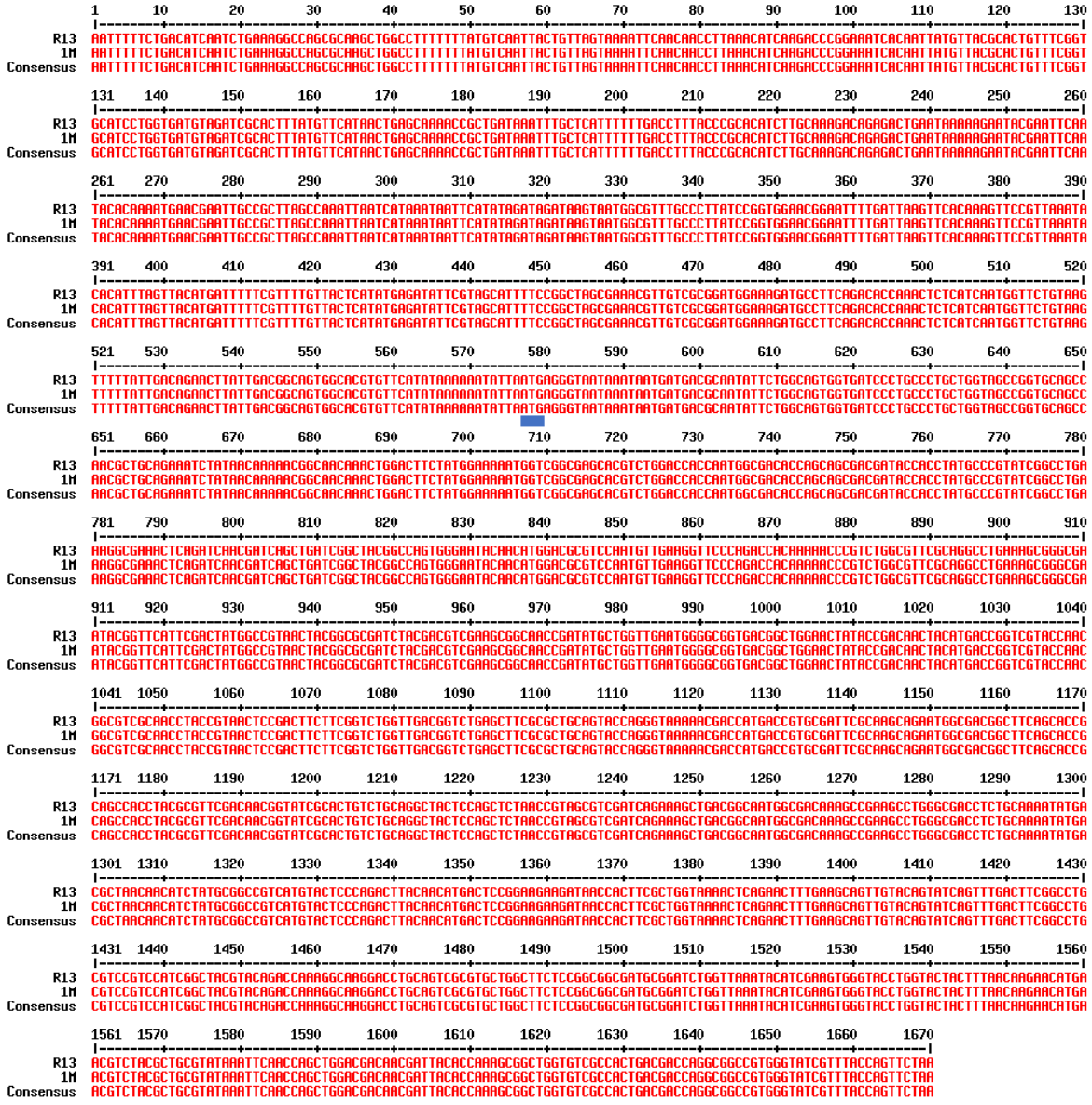

Figure 30. Intergenic and coding regions of *ompK36* in R13 and 1M. Sequence highlighted in red shows consensus between R13 and 1M. *ompK36* start codon is at position 577 (underlined in blue) with its stop codon at position 1670. The stop codon of the previous gene *rcsD* is at position 1.

4.3. Alternative β-lactam/vaborbactam combinations

As the four mutants 1M, 2M, 64M and 16M showed resistance to meropenem/vaborbactam, a panel of alternative β-lactam partners were tested against them in combination with vaborbactam. The panel of β-lactams was the same as tested against the isogenic Ecl8 strains in chapter 3. We were interested to see if vaborbactam could potentiate the activity of alternative β-lactam antibiotics even in meropenem/vaborbactam resistant mutants, potentially suggesting future clinical application.

4.3.1. Activity of combinations in the presence of CTX-M

The clinical isolate R13, along with being susceptible to meropenem/vaborbactam showed susceptibility to imipenem/vaborbactam and doripenem/vaborbactam (table 22). Vaborbactam was unable to restore susceptibility to ertapenem (which is caused by a combination of CTX-M plus efflux (192)) or cephalosporins apart from ceftazidime, which is a weak CTX-M substrate (244). The same combinations were also tested against the meropenem/vaborbactam resistant derivative of R13, mutant 1M. We can see that even though 1M is resistant to imipenem (but not doripenem) in the absence of vaborbactam, vaborbactam restores imipenem susceptibility. However, the mutation in 1M prevents vaborbactam from restoring ceftazidime susceptibility as well as meropenem susceptibility (Table19).

Antibiotic (concentration µg in disc)	**R13 (CTX-M)**	**R13 (CTXM) +Vab**	**1M (CTX-M)**	**1M (CTX-M) +Vab**
ERT (10)	15.0	18.0	6.0 (-9.0)	6.0 (-12.0)
MEM (10)	22.0	27.0	20.0 (-2.0)	22.0 (-5.0)
IPM (10)	28.0	30.0	18.7 (-9.3)	27.0 (-3.0)
DOR (10)	25.0	27.0	23.0 (-2.0)	24.0 (-3.0)
CAZ (30)	10.0	21.0	6.7 (-3.3)	15.7 (-5.3)
CXM (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CTX (30)	6.0	16.0	6.0 (NC)	6.0 (-10.0)
FEP (30)	9.0	20.0	6.0 (-3.0)	6.0 (-14.0)
CRO (30)	6.0	15.0	6.0 (NC)	6.0 (-9)

Table 22. Antimicrobial disc susceptibility testing of clinical isolate R13 and derived mutant 1M against a panel of β-lactams. White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation Adjusted Ca^{2+} Mueller Hinton. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent.

4.3.2. Activity of combinations in the presence of KPC

Vaborbactam can efficiently restore susceptibility to ertapenem, meropenem, imipenem, doripenem, ceftazidime and ceftriaxone in the KPC producing clinical isolate R30 (table 23). In the meropenem/vaborbactam resistant mutant derivative 2M, vaborbactam can only restore susceptibility of imipenem and ceftriaxone. Similarly, to the CTX-M producing mutant 1M, therefore, the KPC producing mutant 2M has lost ceftazidime/vaborbactam susceptibility as well as meropenem/vaborbactam susceptibility. This could indicate that OmpK36 downregulation – the likely cause of meropenem/vaborbactam resistance in 1M and 2M is an important indicator of ceftazidime/vaborbactam non-susceptibility. As with 1M, imipenem/vaborbactam susceptibility was retained in 2M, suggesting that this combination has potential for dealing with meropenem/vaborbactam resistant mutants of isolates producing KPC or CTX-M.

Antibiotic (concentration µg in disc)	R30 (KPC)	R30 (KPC) + Vab	2M (KPC)	2M (KPC) +Vab
ERT (10)	6.0	23.0	6.0 (NC)	8.0 (-15)
MEM (10)	6.0	25.0	6.0 (NC)	14.0 (-11.0)
IPM (10)	6.0	27.7	6.0 (NC)	24.3 (-3.4)
DOR (10)	6.0	26.7	6.0 (NC)	20.0 (-6.7)
CAZ (30)	6.0	22.0	6.0 (NC)	14.7 (-7.3)
CXM (30)	6.0	11.0	6.0 (NC)	6.0 (NC)
CTX (30)	8.7	25.0	6.0 (-2.7)	22.0 (-3.0)
FEP (30)	7.0	24.3	6.0 (-1.0)	20.7 (-3.6)
CRO (30)	7.0	25.3	6.0 (-1.0)	23.0 (-2.3)

Table 23. Antimicrobial disc susceptibility testing of clinical isolate R30 and derived mutant 2M against a panel of β-lactams White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation Adjusted Ca^{2+} Mueller Hinton. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent.

4.3.3. Activity of combinations in the presence of OXA-232

Vaborbactam has been reported as a poor class D β-lactamase inhibitor in combination with meropenem. The results in section 4.1. and chapter 3, showed this to be true, where vaborbactam was unable to restore meropenem susceptibility in the OXA-232 producing strains R4, R11 and R21 OXA. We were interested to see whether vaborbactam could restore susceptibility to any of the β-lactams tested. Table 24 shows that R11 (which produces both OXA-232 and CTX-M-15) and the mutant derivative selected for very high meropenem/vaborbactam MICs, 64M are resistant to all β-lactams tested in the absence of vaborbactam. However, vaborbactam was able to potentiate the activity of ceftazidime and restore susceptibility in both R11 and 64M. Therefore, the addition of ceftazidime to the meropenem/vaborbactam effectively extends meropenem/vaborbactam activity to include class D β-lactamases.

Antibiotic (concentration µg in disc)	R11 (OXA-232)	R11 (OXA-232) +Vab	64M (OXA-232)	64M (OXA-232) +Vab
ERT (10)	6.0	6.0	6.0 (NC)	6.0 (NC)
MEM (10)	11.0	15.3	6.7 (-3.3)	6.3 (-9.0)
IPM (10)	12.7	11.3	7.0 (-5.7)	7.0 (-4.3)
DOR (10)	10.7	12.7	6.3 (-4.4)	6.0 (-6.7)
CAZ (30)	16.3	23.0	15.7 (-0.6)	24.3 (+1.3)
CXM (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CTX (30)	6.3	17.0	6.0 (-0.3)	15.7 (-1.3)
FEP (30)	12.0	16.3	9.3 (-2.7)	21.7 (+5.4)
CRO (30)	6.7	18.3	6.0 (-0.3)	17.0 (-1.3)

Table 24. Antimicrobial disc susceptibility testing of clinical isolate R11 and derived mutant 64M against a panel of β-lactams White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Mueller Hinton agar. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent.

Since the OXA-232 producing isolate R11 also produces CTX-M15, which is a better target for vaborbactam, it is likely that this is the real reason for ceftazidime resistance, not OXA-232. To test this hypothesis, the same panel of β-lactams was tested against the R21 OXA and the higher meropenem/vaborbactam MIC mutant 16M. R21 OXA and its mutant 16M only produces OXA-232 and does not produce CTX-M. The resulting antimicrobial disc susceptibility results (table 25) shows that R21 and indeed its mutant 16M are susceptible to ceftazidime and ceftazidime/vaborbactam. This shows that the CTX-M-15 enzyme present in R11 is indeed causing ceftazidime resistance in the absence of vaborbactam. Therefore, the reason vaborbactam is able to rescue ceftazidime resistance in R11 is due to its inhibitory effects on CTX-M-15 not on OXA-232. While R21 OXA was also susceptible to Cefotaxime, Cefepime and Ceftriaxone, both in the presence and absence of vaborbactam. The mutant 16M was only susceptible to Ceftazidime and Ceftriaxone.

Whilst the CTX-M-15 producing mutant 64M remained susceptible to ceftazidime/vaborbactam, the CTX-M-15 producing mutant 1M was resistant to ceftazidime/vaborbactam. As both, 1M and 64M have *ramR* mutation, *ompK35* loss of function and OmpK36 down-regulation, we would expect them both to have the same ceftazidime/vaborbactam susceptibility phenotype. The reason that *ompK36* loss in 64M does not stop vaborbactam from protecting ceftazidime could be due to an underlying difference in permeability, however. Both 1M and 64M contain a truncated non-functional *ompK35*. 1M contains a truncated *ramR,* whereas 64M contains a *ramR* point mutation (M184V). Although, both mutations have previously been shown to hyper-express *acrB* efflux pump gene by qRT-PCR, we hypothesise that the truncated *ramR* present in 1M to be a stronger mutation compared to the point mutations present in 64M. We believe this to be the key difference between the two mutants and leads to the inability of *ompK36* loss to give ceftazidime/vaborbactam resistance in 64M. Alternatively, there could be a difference in the

production level of CTX-M-15 in the two mutants, meaning the ceftazidime hydrolysing potential of 1M is greater than that of 64M (226).

Antibiotic (concentration µg in disc)	R21 (OXA-232)	R21 (OXA-232) +Vab	16M (OXA-232)	16M (OXA-232) +Vab
ERT (10)	14.0	18.0	6.0 (-8.0)	6.0 (-12.0)
MEM (10)	21.0	21.0	6.7 (-14.3)	6.3 (-14.7)
IPM (10)	22.0	20.3	9.0 (-13.0)	8.0 (-12.3)
DOR (10)	19.0	19.0	10.0 (-9.0)	10.0 (-9.0)
CAZ (30)	24.0	28.0	23.0 (-1.0)	26.0 (-2.0)
CXM (30)	16.0	16.0	10.0 (-6.0)	12.0 (-4.0)
CTX (30)	26.0	30.7	22.0 (-4.0)	24.3 (-6.4)
FEP (30)	26.0	26.0	22.0 (-4.0)	23.0 (-3.0)
CRO (30)	25.0	25.3	25.0 (NC)	27.0 (+1.7)

Table 25. Antimicrobial disc susceptibility testing of clinical isolate R21 OXA and derived mutant 16M against a panel of β-lactams White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation Adjusted Ca^{2+} Mueller Hinton. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent.

4.4. Ceftazidime, Imipenem and Doripenem as alternative partners for vaborbactam

Ceftazidime was identified as a potential partner for vaborbactam against the OXA-232 producers R11 (OXA-232, CTX-M15) and R21 OXA-232 even in mutants with OmpK36 downregulation. Imipenem was identified as a potential partner for CTX-M-15 or KPC-3 producers, even with highly impermeable envelopes and with OmpK36 downregulation, which are ceftazidime/vaborbactam non-susceptible. Doripenem/vaborbactam was also identified as a potential combination to combat highly impermeable isolate R13 (CTX-M-15) and its OmpK36 downregulated mutant 1M. These combinations are not clinically available, with meropenem/vaborbactam being the only available combination. However, it is possible

for clinicians to administer one of these agents at the same time as meropenem/vaborbactam, essentially creating these combinations. To ensure the effectiveness of these combinations, therefore, against our highly resistant clinical isolates and mutants, chequerboard MICs of ceftazidime, imipenem or doripenem each separately put in combination with meropenem/vaborbactam were carried out in the four meropenem/vaborbactam non-susceptible mutants: 1M (CTX-M-15), 2M (KPC-3), 64M (CTX-M-15, OXA-232) and 16M (OXA-232).

4.4.1. Imipenem shows promise as an alternative partner for vaborbactam against CTX-M producers

The results of the chequerboard MIC assays carried out against the meropenem/vaborbactam mutant 1M is shown in figure 31. Figure 31 A shows that, as predicted using disc susceptibility testing (table 22) 1M is ceftazidime non-susceptible, even in the presence of vaborbactam, which is provided by the meropenem/vaborbactam combination. It is also, as predicted, non-susceptible to meropenem/vaborbactam in the absence of ceftazidime. However, there is a synergistic effect. In the presence of 0.5 mg/L of meropenem (below the breakpoint for clinical non-susceptibility, and so clinically achievable) the MIC of ceftazidime reduces to ≤0.5, which is clinically susceptible. Hence, even though disc testing shows ceftazidime/vaborbactam does not work against this mutant, the clinical use of ceftazidime plus meropenem/vaborbactam should work.

Figure 31 B confirms doripenem susceptibility in the presence of vaborbactam, also as predicted by the disc testing. Indeed, if used in combination with meropenem/vaborbactam the doripenem MIC drops further to ≤0.5 mg/L. Finally, figure 31 C) shows that the MIC of Imipenem is ≤0.5 mg/L with vaborbactam or meropenem/vaborbactam. Whilst synergy may also be present, the chequerboards used did not go to a low enough imipenem concentration to see it. We conclude therefore that whilst ceftazidime/meropenem/vaborbactam and doripenem/meropenem/vaborbactam should work clinically against this highly resistant mutant, the MICs are lowest for imipenem/meropenem/vaborbactam.

A) Ceftazidime (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
0												
0.5												
1												
2												
4												
8												
16												
32												

B) Doripenem (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
0												
0.5												
1												
2												
4												
8												
16												
32												

C) Imipenem (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
0												
0.5												
1												
2												
4												
8												
16												
32												

Figure 31. Chequerboard MICs of R13 1M tested against ceftazidime, doripenem and imipenem. Meropenem/vaborbactam is indicated along the X-axis. Cells filled in grey indicate bacterial growth, cells filled white indicate no bacterial growth. Concentration highlighted in red shows the non-susceptible cut-off (growth above these concentrations indicates non-susceptibility). A) Chequerboard MIC of ceftazidime/vaborbactam Y-axis against meropenem/vaborbactam X-axis. B) Chequerboard MIC of doripenem/vaborbactam Y-axis against meropenem/vaborbactam X-axis. C) Chequerboard MIC of imipenem/vaborbactam Y-axis against meropenem/vaborbactam X-axis. Vaborbactam present at a fixed concentration of 8 mg/L

4.4.2. Ceftazidime, imipenem and doripenem are unable to restore meropenem/vaborbactam resistance in KPC producers

Similarly, ceftazidime, doripenem and imipenem in combination with meropenem/vaborbactam were tested against the KPC-3 meropenem/vaborbactam resistant mutant 2M. The chequerboard MIC results of these three combinations are shown in figure 32. The mutant is resistant to ceftazidime, doripenem and imipenem in combination with vaborbactam alone (zero meropenem), even though the antimicrobial disc susceptibility tests in table 23 showed susceptibility to imipenem/vaborbactam. Unfortunately, against this KPC-3 producing mutant, the MIC of ceftazidime, doripenem or imipenem did not reduce to below the susceptible breakpoint in the presence of a clinically obtainable concentration of meropenem/vaborbactam.

A) Ceftazidime (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.25	0.5	1	2	4	8	16	32	64	128	256
0												
0.5												
1												
2												
4												
8												
16												
32												

B) Doripenem (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.25	0.5	1	2	4	8	16	32	64	128	256
0												
0.5												
1												
2												
4												
8												
16												
32												

C) Imipenem (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.25	0.5	1	2	4	8	16	32	64	128	256
0												
0.5												
1												
2												
4												
8												
16												
32												

Figure 32. Chequerboard MICs of R30 2M tested against ceftazidime, doripenem and imipenem. meropenem vaborbactam is indicated along the X-axis. Cells filled in grey indicate bacterial growth, cells filled white indicate no bacterial growth. Concentration highlighted in red shows the non-susceptible cut-off standard for each antibiotic tested (growth above these concentrations indicates non-susceptibility). A) Chequerboard MIC of ceftazidime/vaborbactam Y-axis against meropenem/vaborbactam X-axis. B) Chequerboard MIC of doripenem/vaborbactam Y-axis against meropenem/vaborbactam X-axis. C) Chequerboard MIC of imipenem/vaborbactam Y-axis against meropenem/vaborbactam X-axis. Vaborbactam present at a fixed concentration of 8 mg/L

4.4.3. Ceftazidime shows promise as an alternative partner for vaborbactam against OXA-232 producers

The chequerboard MIC results of ceftazidime, doripenem and imipenem against the mutant 64M are shown in figure 33. The only combination 64M is susceptible to is ceftazidime/vaborbactam with meropenem or not. Susceptibility to doripenem and imipenem was not achieved at a clinically obtainable concentration of meropenem/vaborbactam.

A) Ceftazidime (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.25	0.5	1	2	4	8	16	32	64	128	256
0												
0.5												
1												
2												
4												
8												
16												
32												

B) Doripenem (X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.25	0.5	1	2	4	8	16	32	64	128	256
0												
0.5												
1												
2												
4												
8												
16												
32												

C) Imipenem(X-axis) versus meropenem (Y axis) in the presence of vaborbactam (all wells)

	0	0.25	0.5	1	2	4	8	16	32	64	128	256
0												
0.5												
1												
2												
4												
8												
16												
32												

Figure 33. Chequerboard MICs of R11 64M tested against Ceftazidime, Doripenem and Imipenem. meropenem vaborbactam is indicated along the X-axis. Cells filled in grey indicate bacterial growth, cells filled white indicate no bacterial growth. Concentration highlighted in red shows the non-susceptible cut-off (growth above these concentrations indicates non-susceptibility). A) Chequerboard MIC of ceftazidime/vaborbactam Y-axis against meropenem/vaborbactam X-axis. B) Chequerboard MIC of doripenem/vaborbactam Y-axis against meropenem/vaborbactam X-axis. C) Chequerboard MIC of imipenem/vaborbactam Y-axis against meropenem/vaborbactam X-axis. Vaborbactam present at a fixed concentration of 8 mg/L

4.5. Characterising ceftazidime/vaborbactam resistance

Of the three antibiotics tested in section 4.4., only imipenem and ceftazidime were shown to be efficacious partners for vaborbactam, or meropenem/vaborbactam. Ceftazidime/vaborbactam was shown to be an alternative combination to combat OXA-232 producers, even if they have OmpK36 downregulated. Although the meropenem/vaborbactam resistant, CTX-M-15 producing, OmpK36 downregulated mutant R13 1M was resistant to ceftazidime/vaborbactam (though susceptible to ceftazidime/meropenem/vaborbactam), the original CTX-M-15 producing R13 isolate was susceptible to ceftazidime/vaborbactam. In order to understand if, and if so, how, ceftazidime/vaborbactam resistance might be achieved, ceftazidime/vaborbactam resistant mutants were selected from the CTX-M-15 producing clinical isolate R13, a CTX-M-15

transformant of the clinical isolate R21 and the meropenem/vaborbactam resistant mutant (64M) derived from the OXA-232/CTXM-15 producing clinical isolate R11.

4.5.1. Ceftazidime/vaborbactam resistant mutants

Three ceftazidime/vaborbactam resistant mutants were selected. These were the R13 ceftazidime/vaborbactam resistant mutant 8CAZ (the number '8' denotes the concentration of ceftazidime the mutant was selected on, 8 µg/ml), the R21 CTX-M mutant 2CAZ and finally the ceftazidime/vaborbactam resistant mutant, derived from R11 64M, 64M8CAZ. The antimicrobial disc susceptibility test results for these three mutants are shown in table 26. All three mutants have indeed lost susceptibility to ceftazidime/vaborbactam. The CTX-M-15 producing ceftazidime/vaborbactam resistant mutants retain susceptibility to imipenem/vaborbactam and doripenem/vaborbactam, but the mutant 64M8CAZ is resistant to all β-lactam/vaborbactam combinations tested. Whole cell proteomics was carried out to try and characterise ceftazidime/vaborbactam resistance. These mutants were also sent off for whole genome sequencing, but unfortunately, due to the time constraints of this project, the whole genome sequencing data of the ceftazidime/vaborbactam mutants 8CAZ and 2CAZ have not been returned to us and cannot be discussed.

A)

Antibiotic (concentration µg in disc)	**R13 (CTX-M)**	**R13 (CTXM) +Vab**	**8CAZ (CTX-M)**	**8CAZ (CTX-M) +Vab**
ERT (10)	15.0	18.0	9.0 (-4.0)	12.0 (-6.0)
MEM (10)	22.0	27.0	20.0 (-3.0)	21.0 (-6.0)
IPM (10)	28.0	30.0	30.0 (+2.0))	32.0 (+2.0)
DOR (10)	25.0	27.0	19.0 (-6.0)	24.0 (-3.0)
CAZ (30)	10.0	21.0	6.0 (-3.7)	16.0 (-5.0)
CXM (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CTX (30)	6.0	16.0	6.0 (NC)	6.0 (-6.0)
FEP (30)	9.0	20.0	6.0 (-3.0)	6.0 (-14.0)
CRO (30)	6.0	15.0	6.0 (NC)	6.0 (-9.0)

B)

Antibiotic (concentration µg in disc)	R21 (CTX-M)	R21 (CTX-M) +Vab	2CAZ (CTX-M)	2CAZ (CTX-M) +Vab
ERT (10)	17.0	25.0	16.0 (-1.0)	24.0 (-1.0)
MEM (10)	31.0	31.0	25.0 (-6.0)	30.0 (-1.0)
IPM (10)	32.7	33.7	30.0 (-2.7)	28.0 (-5.7)
DOR (10)	31.3	31.7	26.0 (-5.3)	27.0 (-4.7)
CAZ (30)	9.0	26.3	6.0 (-3.0)	15.0 (-11.3)
CXM (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CTX (30)	6.0	25.0	6.0 (NC)	6.0 (-19.0)
FEP (30)	6.0	14.0	6.0 (NC)	6.0 (-8.0)
CRO (30)	6.0	22.0	6.0 (NC)	6.0 (-16.0)

C)

Antibiotic (concentration µg in disc)	64M (OXA-232, CTX-M)	64M (OXA-232, CTX-M) +Vab	64M8CAZ (OXA-232, CTX-M)	64M8CAZ (OXA-232, CTX-M) +Vab
ERT (10)	6.0	6.0	6.0 (NC)	6.0 (NC)
MEM (10)	6.7	6.3	7.0 (+0.3)	7.0 (+0.7)
IPM (10)	7.0	7.0	6.0 (-1.0)	6.0 (-1.0)
DOR (10)	6.3	6.0	7.0 (+0.3)	7.0 (+1.0)
CAZ (30)	15.7	24.3	8.0 (-7.7)	19.0 (-5.3)
CXM (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CTX (30)	6.0	15.7	6.0 (NC)	6.0 (-9.7)
FEP (30)	9.3	21.7	6.0 (NC)	6.0 (-15.7)
CRO (30)	6.0	17.0	6.0 (NC)	6.0 (-11.0)

Table 26. Antimicrobial disc susceptibility testing of Ceftazidime/vaborbactam resistant mutants. White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation Adjusted Ca^{2+} Mueller Hinton. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent.

4.5.2. Whole cell proteomics of Ceftazidime/vaborbactam mutants

Phenotypically, both the CTX-M-15 Ceftazidime/vaborbactam mutants 2CAZ and 8CAZ show significant upregulation of CTX-M (Uniprot accession Q7AVW6) (tables 27-28) with fold changes of 4.67 and 11.15 respectively. 2CAZ also shows an increase in efflux and decrease in permeability, with a significant up-regulation of AcrA (A6T5M5) and TolC (A6TE24) and significant down-regulation of OmpK36 (A6TBT2). Our hypothesis is that the increase in expression of CTX-M increases ceftazidime hydrolysis activity in the presence of the inhibitory effects of vaborbactam against CTX-M. We were expecting to see a similar proteomics profile in the ceftazidime/vaborbactam mutant 64M8CAZ, since, as well as producing OXA-232 carbapenemase, it also produces CTX-M-15, which could, presumably be overproduced. However, this mutant does not show an increase in production of CTX-M (table 29). Instead, this mutant shows down-regulation of OmpK36, NlpD, AcrB and TolC. We have seen that in chapter 3 the mutant, Ecl8-16-1 has a mutation in *nlpD* (Ismah et al, unpublished). This mutant was selected for on the cephalosporin cefuroxime. Therefore down-regulation/loss of NlpD seems to be a novel cephalosporin resistance mechanism. No changes in the open reading frame or promoter region could be found in *nlpD* in 64M8CAZ according to PCR sequencing, and the mechanism of downregulation involved will await genome sequence results however an IS element was found in the promoter region of *ompK36.* Analysis of this IS element using IS finder, showed it was IS*Kp*n14 which belongs to the IS1 family of IS elements (245, 246). As OmpK36 is already lost in 64M we do not think the additional IS element present in 64M8CAZ to be responsible for the antimicrobial disc susceptibility profile seen in table 23.

Accession	Description	Fold-change	T-test
A6TFK7	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	2.12	0.021
A6TF25	33 kDa chaperonin	2.20	0.036
A6TI02	3-phosphoserine phosphatase	2.15	0.032
A6TD51	7-carboxy-7-deazaguanine synthase	>20	<0.001
Q6SJ71	AAC(6')-Ib	5.94	0.009
A6TBY3	Acetate kinase	2.11	0.021
A6T7U1	Activator of ntrL	>20	<0.001
A6T9W8	Adenosine deaminase	2.59	0.023
A6T5N7	Adenylate kinase	2.37	0.036
A6T679	Alkyl hydroperoxide reductase	2.15	0.042
A0A0N6WZQ7	Anaerobic nitric oxide reductase flavorubredoxin	7.35	0.005
A6T5I1	ATP-dependent Clp protease	2.09	0.012
A6TCK0	ATP-dependent protease	2.30	0.032
A6T634	ATP-dependent serine activating enzyme	>20	<0.001
A6TGR3	Bifunctional purine biosynthesis protein	>20	<0.001
A6T4I0	Carbamoyl-phosphate synthase	4.40	0.009
A6TA21	Carbonic anhydrase	2.33	0.044
A6T4F4	Chaperone protein	2.05	0.041
A6T5N6	Chaperone protein	2.04	0.037
A0A0C4Y240	Chromosome partitioning protein	>20	<0.001
A6TAT4	Cold shock protein E	2.44	0.023
A6T8N9	Conserved protein	>20	<0.001
Q7AVW6	CTX-M-1	4.67	0.038
A6TCF1	Cysteine desulfurase	2.16	0.016
A6TC50	Cysteine synthase	2.48	0.012
A6TB70	Cysteine transport protein	2.38	0.036
A6T5R2	Cysteine--tRNA ligase	2.30	0.035
A6T5H5	Cytochrome o ubiquinol oxidase subunit	2.20	0.018
A6T4N5	D-alanine--D-alanine ligase	2.77	0.002
A6T4I4	Dihydrofolate reductase	>20	<0.001
A0A0A2RH67	Dihydrolipoyl dehydrogenase	2.67	0.019
A6TGP1	DNA-directed RNA polymerase	10.12	0.009
A6TGP0	DNA-directed RNA polymerase subunit	11.64	0.010
A0A0A2R562	Elongation factor G	>20	<0.001
A6T6C9	Flavodoxin	3.53	0.015
W8SYC3	Formate dehydrogenase	>20	<0.001
A6T6K8	Formimidoylglutamase	2.76	0.043
A6TBJ6	Fructose-bisphosphate aldolase	2.31	0.024
A6T6C1	Glucosamine-6-phosphate deaminase	2.20	0.013
A6TB22	Glucose-6-phosphate 1-dehydrogenase	2.39	0.030
A6TEN3	Glutamate synthase	>20	<0.001
A6T4V8	Glutamate-1-semialdehyde 2,1-aminomutase	2.11	0.024
A6TFH5	Glycine--tRNA ligase	2.66	0.034
A6TCD0	GTPase	>20	<0.001
A6THD8	Inorganic pyrophosphatase	2.32	0.036

A6TGG1	Ketol-acid reductoisomerase	2.63	0.044
A6T6A3	Leucine--tRNA ligase	2.54	0.020
A6TAY5	L-serine deaminase	2.42	0.016
A6T4X0	Methionine aminopeptidase	2.17	0.030
A6T4E9	Molybdenum cofactor biosynthesis protein	84.24	0.026
A6T7A5	Multifunctional: proline dehydrogenase	7.20	0.012
A6TBW4	NADH dehydrogenase	>20	<0.001
A6T6Y8	Outer-membrane lipoprotein carrier protein	2.02	0.026
K9LL58	OXA-1	>20	<0.001
D7EZP3	ParB	>20	<0.001
A6TEY5	Peptidyl-prolyl cis-trans isomerase	2.84	0.031
A6TBY6	Phosphodiesterase	>20	<0.001
A6TF09	Phosphoglycolate phosphatase	>20	<0.001
A6TAM2	Pleiotrophic regulation of anaerobic respiration	>20	<0.001
A6TFK4	Protein-export protein SecB	>20	<0.001
A6T7X7	Pseudouridine synthase	2.73	0.030
A6T7G1	PTS family enzyme IIC/enzyme IIB	2.68	0.017
A6TEM0	Putative ATP-binding component of a transport system	2.21	0.039
A6TAP8	Putative GTP-binding protein	2.02	0.024
A6TFG2	Putative outer membrane protein	4.02	0.009
A6T7C0	Putative oxidoreductase component	>20	<0.001
A6TBX9	Putative phosphatase	>20	<0.001
A6TA14	Putative uncharacterized protein	>20	<0.001
A6TDW6	Putative uncharacterized protein	>20	<0.001
A6TGL4	Putative uncharacterized protein	>20	<0.001
A6T8N5	Putative uncharacterized protein	2.38	0.029
A6TFG8	Putative uncharacterized protein	2.05	0.044
A6TGI5	Putative uncharacterized protein hemY	2.11	0.046
A6T6Z5	Pyruvate formate lyase activating enzyme	2.59	0.031
A6TBT9	Ribonucleotide-diphosphate reductase	2.36	0.020
A6TEI6	Ribosome-binding factor	>20	<0.001
A6T4J7	RNA polymerase-associated protein	2.50	0.032
A6T4T4	RNA polymerase-binding transcription factor	2.39	0.033
A6T7E5	RNase E	5.87	0.022
A0A0A2RAJ9	Serine hydroxymethyltransferase	>20	<0.001
C3TGI7	Serine--tRNA ligase	>20	<0.001
A6TEN7	Stringent starvation protein	2.03	0.043
A6TA04	Superoxide dismutase	2.68	0.013
C4NV37	TEM-1	>20	<0.001
A6TG65	Thiol:disulfide interchange protein	2.11	0.045
A6TAI7	Threonine--tRNA ligase	2.21	0.047
A6TDG3	Thymidylate synthase	>20	<0.001
A6TEK0	Transcription elongation factor	3.06	0.013
A6TGN4	Transcription termination/antitermination protein	2.46	0.007
C3T7P7	Translation initiation factor	2.42	0.037
A6TEI7	Translation initiation factor	2.18	0.038

A6TG45	tRNA uridine 5-carboxymethylaminomethyl modification enzyme	2.29	0.013
A6TDQ2	tRNA-modifying protein	3.13	0.043
A6T7K3	tRNA-specific 2-thiouridylase	>20	<0.001
A6TGL3	Ubiquinone/menaquinone biosynthesis methyltransferase	3.33	0.015
A6THE7	UPF0307 protein	>20	<0.001
A6T4X3	Uridylate kinase	4.24	0.012
A6T4T0	3-methyl-2-oxobutanoate hydroxymethyltransferase	<0.05	<0.001
E2QPI4	3-oxoacyl-(Acyl-carrier-protein) synthase	<0.05	<0.001
A6T4G3	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	<0.05	<0.001
B0CN74	60 kDa chaperonin	<0.05	<0.001
A6TF98	Bifunctional polymyxin resistance protein	<0.05	<0.001
A6TGQ7	DNA-binding protein	0.41	0.010
A6TFP8	DNA-directed RNA polymerase	<0.05	<0.001
Q798T6	FyuA	<0.05	<0.001
A0A0A2RBF5	Glyceraldehyde-3-phosphate dehydrogenase	<0.05	<0.001
W8T014	GTPase	<0.05	<0.001
C3SZX2	Histidine--tRNA ligase	<0.05	<0.001
A6TBJ8	Hydroxyethylthiazole kinase	<0.05	<0.001
A6TAH5	Lipoprotein	<0.05	<0.001
A6T5S9	Lysine decarboxylase 1	<0.05	<0.001
A0A061YB63	Lysine--tRNA ligase	<0.05	<0.001
A6TGU6	Maltoporin 2	0.45	0.004
A6TCU9	Multidrug resistance secretion protein	<0.05	<0.001
A6TAE1	Murein lipoprotein	<0.05	<0.001
A6T9Z9	N-ethylmaleimide reductase	<0.05	<0.001
A0A0G3B7F3	OXA-1	<0.05	<0.001
A6TG32	Phosphate-binding protein	0.27	0.001
A6TBJ7	Phosphomethylpyrimidine kinase	<0.05	<0.001
A6TGQ1	Phosphomethylpyrimidine synthase	<0.05	<0.001
A6TCB4	Phosphoribosylglycinamide formyltransferase	<0.05	<0.001
A6THT4	Putative aldehyde dehydrogenase	<0.05	<0.001
A6TFP1	Putative alpha helix protein	0.50	0.019
A6T985	Putative LysM domain	0.46	0.003
A6T9B7	Putative NADH	<0.05	<0.001
A6TBQ4	Putative permease	<0.05	<0.001
A6TE18	Putative uncharacterized protein	<0.05	<0.001
A6TED7	Putative uncharacterized protein	<0.05	<0.001
A6TGV9	Quinone oxidoreductase	<0.05	<0.001
A6T7J8	Response regulator	<0.05	<0.001
A6T7Q6	Scaffolding protein	<0.05	<0.001
X2JHQ6	TEM	<0.05	<0.001
A6T4K6	Thiamin-binding periplasmic protein	<0.05	<0.001
A6TGP6	Thiamine biosynthesis protein	<0.05	<0.001
A6TGP9	Thiamine biosynthesis protein	<0.05	<0.001
A6TGQ0	Thiamine-phosphate synthase	<0.05	<0.001

A6TGP7	Thiazole synthase	<0.05	<0.001
A0A0A2R7D6	Transaldolase	<0.05	<0.001
A6T4M9	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	<0.05	<0.001
A6TCJ2	Uracil-DNA glycosylase	<0.05	<0.001

Table 27. Whole cell proteomics results of R13 compared to the derived mutant 8CAZ. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 8CAZ. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

Accession	Description	Fold-change	T-test
A6TI02	3-phosphoserine phosphatase	>20	<0.001
A6TFX3	Acetolactate synthase	>20	<0.001
A6T5M5	AcrA	5.44	0.043
A6TB47	Alpha,alpha-trehalose-phosphate synthase	>20	<0.001
A6T6W2	ATP-binding component of 3rd arginine transport system	>20	<0.001
A6TBL5	Beta-D-glucoside glucohydrolase	>20	<0.001
W1DMB9	Bifunctional purine biosynthesis protein	>20	<0.001
Q7AVW6	CTX-M-1	11.15	0.017
A6T702	Cytidylate kinase	>20	<0.001
A6THZ6	Deoxyribose-phosphate aldolase	>20	<0.001
A6T739	Dihydroorotate dehydrogenase	>20	<0.001
A6TCT3	DNA-bending protein	>20	<0.001
A6T9X6	Electron transport complex protein	>20	<0.001
A6TF60	Gamma-glutamyltranspeptidase	>20	<0.001
A6TEN3	Glutamate synthase	>20	<0.001
A6T9Y3	Glutathionine S-transferase	>20	<0.001
C3SZX2	Histidine--tRNA ligase	>20	<0.001
A6TCF4	Inositol monophosphatase	>20	<0.001
A6TEU4	Large-conductance mechanosensitive channel	>20	<0.001
A6TC22	Long-chain fatty acid transport protein	>20	<0.001
A6TH24	Low-affinity transport system; proline permease II	>20	<0.001
A6T6S5	Molybdopterin biosynthesis protein	>20	<0.001
A6T6C0	N-acetylglucosamine-6-phosphate deacetylase	>20	<0.001
A6TBW4	NADH dehydrogenase subunit	>20	<0.001
A6T9P4	N-hydroxyarylamine O-acetyltransferase	>20	<0.001
A6TD34	NlpD	6.73	0.027
A6TC39	Nucleoside transport protein	>20	<0.001

A6TF28	OmpR	>20	<0.001
A6TDX3	Ornithine decarboxylase isozyme	>20	<0.001
A6T821	Periplasmic murein tripeptide	>20	<0.001
A6TAZ0	PTS enzyme	>20	<0.001
A6T6B4	Putative ATP-binding protein in pho regulon	>20	<0.001
A6TBL4	Putative glycine/betaine/choline transport protein	>20	<0.001
A6THT6	Putative synthesis protein	>20	<0.001
A6TCJ3	Putative tRNA/rRNA methyltransferase	>20	<0.001
A6T6Z8	Putative uncharacterized protein	>20	<0.001
A6TBY0	Putative uncharacterized protein	>20	<0.001
A6TCT6	Putative uncharacterized protein	>20	<0.001
A6TE01	Putative uncharacterized protein	>20	<0.001
A6THQ0	Putative uncharacterized protein	>20	<0.001
A6T6Z5	Pyruvate formate lyase activating enzyme	>20	<0.001
A0A0A2RNF5	Pyruvate kinase	>20	<0.001
A6TFN5	Ribonuclease PH	>20	<0.001
A6TES6	Ribosomal protein L11 methyltransferase	>20	<0.001
A6T6T1	Ribosomal protein S12 methylthiotransferase	>20	<0.001
A6THY6	Ribosomal RNA small subunit methyltransferase	>20	<0.001
A6TEI9	Ribosome maturation factor	>20	<0.001
A6TEI6	Ribosome-binding factor	>20	<0.001
A0A0A2R1S6	RNA-binding protein	>20	<0.001
A6T538	Succinate-semialdehyde dehydrogenase	>20	<0.001
C4NV37	TEM-1	6.42	0.007
A6TAK7	Thymidine kinase	>20	<0.001
A6TE24	TolC	3.69	0.022
A6TAI6	Translation initiation factor	>20	<0.001
C3T7P7	Translation initiation factor	>20	<0.001
A6TAV8	Trehalase, periplasmic	>20	<0.001
A6T7K3	tRNA-specific 2-thiouridylase	>20	<0.001
A6TGH5	UDP-N-acetyl-D-mannosaminuronate dehydrogenase	>20	<0.001
A6TGW6	UvrABC system protein	>20	<0.001
A6TBT2	OmpK36	0.45	0.035

Table 28. Whole cell proteomics results of R21 CTX-M compared to the derived mutant 2CAZ. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 2CAZ. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

Accession	Description	Fold-change	T-test
A6TC02	Acetyl-coenzyme A carboxylase carboxyl transferase	>20	<0.001
A6TEB5	Autoinducer 2-binding protein	>20	<0.001
A6TBL5	Beta-D-glucoside glucohydrolase	>20	<0.001
E2QPY3	Dual-specificity RNA methyltransferase	>20	<0.001
E2QJB4	Formate C-acetyltransferase	>20	<0.001
A6T9T4	Gamma-aminobutyraldehyde dehydrogenase	>20	<0.001
A6TCU5	Glycine/betaine/proline transport protein	>20	<0.001
A6TCD0	GTPase Der OS=Klebsiella pneumoniae	>20	<0.001
A6THJ2	Inositol 2-dehydrogenase	>20	<0.001
A6THJ4	Inosose dehydratase	>20	<0.001
A0A0A5SJ80	Leucine-responsive transcriptional	>20	<0.001
A6T5S8	Lysine/cadaverine transport protein	>20	<0.001
C3T0K2	Malate dehydrogenase	>20	<0.001
A6TBW4	NADH dehydrogenase subunit	>20	<0.001
A6TBW7	NADH-quinone oxidoreductase	>20	<0.001
A0A0U1YUK9	OXA-181	>20	<0.001
A6TF09	Phosphoglycolate phosphatase	>20	<0.001
A6TDI6	Putative ABC transporter	>20	<0.001
A6THJ0	Putative carbohydrate kinase	>20	<0.001
A6TG26	Putative xylanase	>20	<0.001
A6TB55	Ribose-5-phosphate isomerase	>20	<0.001
C3TPL7	Ribosome-recycling factor	>20	<0.001
A0A0A2RAJ9	Serine hydroxymethyltransferase	>20	<0.001
A6TD51	7-carboxy-7-deazaguanine synthase	<0.05	<0.001
A6TES2	Acetyl-CoA carboxylase	<0.05	<0.001
C3T1Y2	Acetyl-coenzyme A carboxylase carboxyl transferase	<0.05	<0.001
A6T5M5	AcrA	0.24	0.001
A6T5M4	AcrB	0.25	0.007
A6TF82	ATP-binding component	<0.05	<0.001
A6TF98	Bifunctional polymyxin resistance protein	<0.05	<0.001
A6T4N7	Cell division protein	<0.05	<0.001
A6TG04	Chromosomal replication initiator protein	<0.05	<0.001
A6TBY9	D-erythro-7,8-dihydroneopterin tri P epimerase	<0.05	<0.001
A6T5I3	DNA-binding protein HU-beta	<0.05	<0.001
A6TAW6	Fatty acid metabolism regulator protein	<0.05	<0.001

A6T7S2	Glutamate dehydrogenase	<0.05	<0.001
A6TA02	Glutaredoxin	<0.05	<0.001
A6TAH5	Lipoprotein	<0.05	<0.001
A0A061YB63	Lysine--tRNA ligase	<0.05	<0.001
A6TD34	NlpD	0.14	0.021
A6T8K6	N-succinylarginine dihydrolase	<0.05	<0.001
C9E714	OmpK35	<0.05	<0.001
A6TBT2	OmpK36	<0.05	<0.001
A6T6Q8	OmpX	0.12	0.003
A6TCA0	Outer membrane protein assembly factor BamC NlpB	0.20	0.006
A6T6Y8	Outer-membrane lipoprotein	<0.05	<0.001
M4JTK1	OXA-232	0.33	0.005
A0A0U1XY39	OXA-48	0.33	0.020
A6TGQ1	Phosphomethylpyrimidine synthase	<0.05	<0.001
A6TEL4	Putative ATP-binding component	<0.05	<0.001
A6T5H7	Putative lipoprotein	<0.05	<0.001
A6TEL2	Putative transport protein	<0.05	<0.001
A6TCV7	Putative uncharacterized protein	<0.05	<0.001
A6T5B7	Recombination-associated protein	<0.05	<0.001
A6T6Y6	Regulator for leucine	<0.05	<0.001
A6TGB9	Regulator of ribonuclease activity	<0.05	<0.001
A6T742	Ribosomal RNA large subunit methyltransferase	<0.05	<0.001
A6TEM7	Sigma cross-reacting protein	<0.05	<0.001
A6TEE8	Tartronate semialdehyde reductase	<0.05	<0.001
A6TGP6	Thiamine biosynthesis	<0.05	<0.001
A6TGP9	Thiamine biosynthesis protein	<0.05	<0.001
A6TGP7	Thiazole synthase	<0.05	<0.001
E2QE61	TolC	0.31	<0.001
A6TE24	TolC	0.20	0.003
A6T7Y3	Transcriptional regulator for cysteine	<0.05	<0.001
A6T4W7	UPF0325 protein	<0.05	<0.001

Table 29. Whole cell proteomics results of R11 64M compared to the derived mutant 64M8CAZ. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 64M8CAZ. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

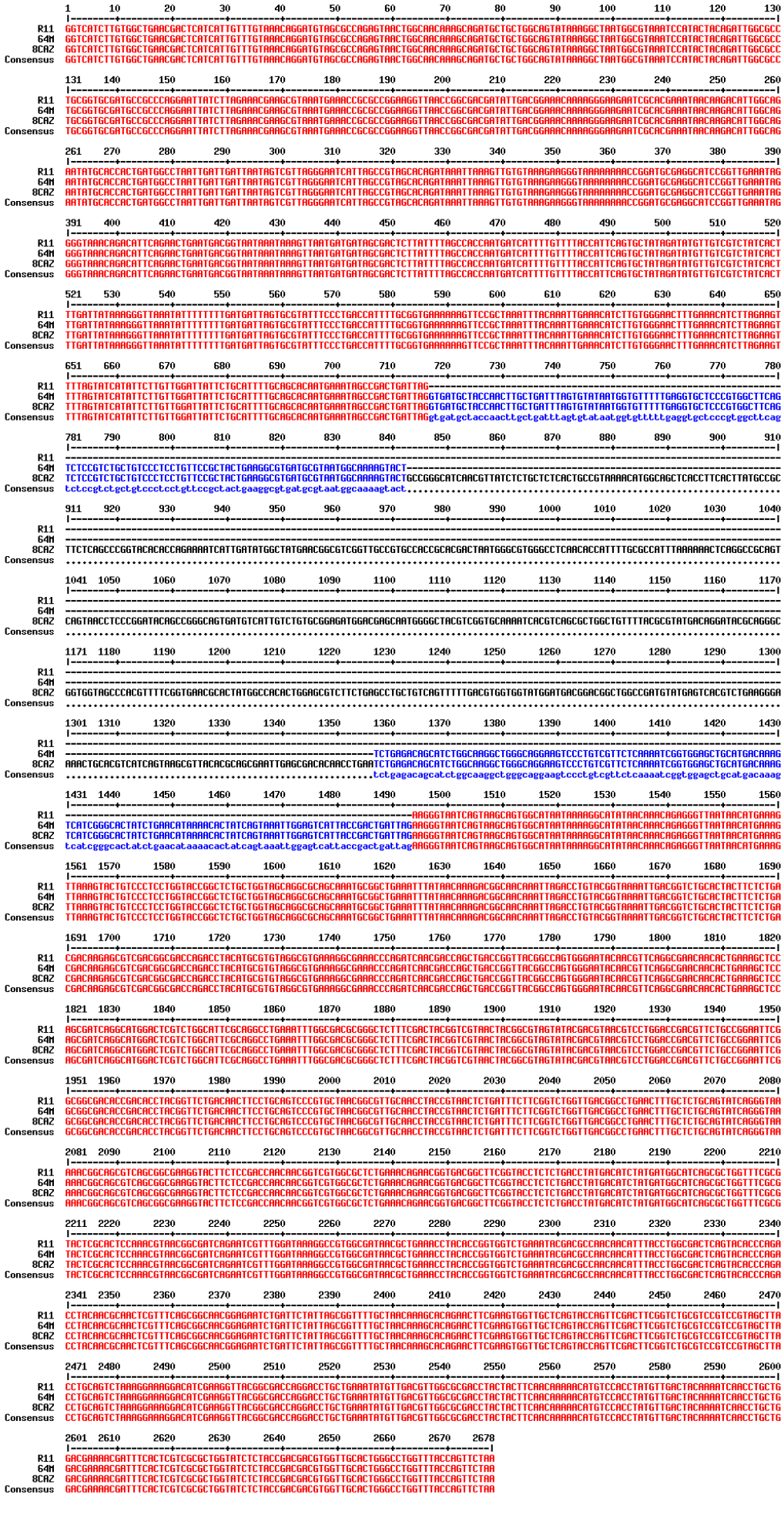

Figure 33. Intergenic and coding regions of *ompK36* in R11, 64M and 64M8CAZ. Sequence highlighted in red shows consensus between R11, 64M and 64M8CAZ. Sequence highlighted in blue indicates the 263bp transposase introduced into 64M. Sequence highlighted in black shows a further 513bp insertion sequence, inserted directly into the middle of the transposase present in 64M.

4.5.3. Imipenem/vaborbactam resistance

The clinical isolate R13 as well as its meropenem/vaborbactam resistant mutant 1M remained susceptible to imipenem/vaborbactam as shown by the checkerboard MICs in figure 13. Ceftazidime/vaborbactam resistant mutants of R13 and R21 (CTX-M) also retained imipenem/vaborbactam susceptibility, as shown above. Therefore, an attempt was made to select for an imipenem/vaborbactam resistant mutant, derived from the meropenem/vaborbactam and ceftazidime/vaborbactam resistant mutant derivative of R13 (CTX-M) 1M. The antimicrobial disc susceptibility tests of the resulting mutant 1 Imi (selected for on 1 µg/ml of imipenem) is shown in table 30. The mutant 1 Imi has lost susceptibility to imipenem/vaborbactam as well as doripenem/vaborbactam. This mutant is now resistant to all β-lactam/vaborbactam combinations tested. Whole cell proteomics was carried out to try and characterise imipenem/vaborbactam resistance. These mutants were also sent off for whole genome sequencing, but unfortunately, the data have not been returned in time to be discussed. The proteomics data (table 31) show a number of differences, but none suggest an obvious mechanism of resistance. Once the whole genome sequence data are available it may become clearer what the mechanism is, and it may be novel.

Antibiotic (concentration µg in disc)	1M (CTX-M)	1M (CTX-M) +Vab	1 Imi (CTX-M)	1 Imi (CTX-M) +Vab
ERT (10)	6.0	6.0	6.0 (NC)	6.0 (NC)
MEM (10)	20.0	22.0	14.0 (-6.0)	15.0 (-7.0)
IPM (10)	18.7	27.0	19.0 (+0.3)	20.0 (-7.0)
DOR (10)	23.0	24.0	15.0 (-8.0)	20.0 (-4.0)
CAZ (30)	6.7	15.7	6.0 (-0.7)	12.0 (-3.7)
CXM (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CTX (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
FEP (30)	6.0	6.0	6.0 (NC)	6.0 (NC)
CRO (30)	6.0	6.0	6.0 (NC)	6.0 (NC)

Table 30. Antimicrobial disc susceptibility testing of 1M and derived mutant 1 Imi against a panel of β-lactams White, susceptible; Red, non-susceptible. Disc susceptibility assays were performed on Cation Adjusted Ca^{2+} Mueller Hinton. Assays were performed according to CLSI guidelines in triplicate. Values in table represent the mean of the three repetitions adjusted to the nearest one decimal point. Numbers in brackets represent the difference in zone diameter compared to the parental strain. 'NC' denotes No Change in zone diameter compared to the parent.

Accession	Description	Fold-change	T-test
A6T7U1	Activator of ntrL gene	>20	<0.001
A6TEM8	Aerobic respiration sensor-response protein; histidine protein kinase/phosphatase, sensor for arcA	>20	<0.001
A6T6Z1	Anaerobic dimethyl sulfoxide (DMSO) reductase	>20	<0.001
A6TGS0	B12-dependent methionine synthase	>20	<0.001
A6TAJ2	Conserved protein, protein kinase-like protein	>20	<0.001
A6TDT2	D-erythrose-4-phosphate dehydrogenase	>20	<0.001
A6TGP0	DNA-directed RNA polymerase	7.27	0.018
A6TGP1	DNA-directed RNA polymerase	3.93	0.028
A0A0A2R562	Elongation factor G	>20	<0.001
E2QJB4	Formate C-acetyltransferase	>20	<0.001
A6TH73	Fumarate reductase	3.58	0.022
A6TEN3	Glutamate synthase	>20	<0.001
A6TE32	Glutamate-ammonia-ligase adenylyltransferase	>20	<0.001

A6TCD0	GTPase	>20	<0.001
A6TD70	L-serine deaminase	2.62	0.032
A6T8N7	NAD(P) transhydrogenase subunit	>20	<0.001
A6T8N6	NAD(P) transhydrogenase subunit	2.16	0.004
A6TG75	Phosphatase	>20	<0.001
A6TD69	Probable serine transporter	2.32	0.034
A6THJ1	Putative acetolactate synthase	>20	<0.001
A6TE06	Putative alcohol dehydrogenase	>20	<0.001
A6THI7	Putative aldehyde dehydrogenase	>20	<0.001
A6TDB9	Putative uncharacterized protein	>20	<0.001
A6TBY1	Putative uncharacterized protein	>20	<0.001
A6TC30	Putative uncharacterized protein	>20	<0.001
A6TH72	Succinate dehydrogenase	>20	<0.001
A0A0A5SIW7	Succinate--CoA ligase	>20	<0.001
A6THC4	2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase bifunctional periplasmic protein	<0.05	<0.001
A6T641	2,3-dihydroxybenzoate-AMP ligase	<0.05	<0.001
A6T4T0	3-methyl-2-oxobutanoate hydroxymethyltransferase	<0.05	<0.001
A6TEE9	5-keto-4-deoxy-D-glucarate aldolase	<0.05	<0.001
Q7DL18	Aldehyde-alcohol dehydrogenase	<0.05	<0.001
A6TF98	Bifunctional polymyxin resistance protein ArnA	<0.05	<0.001
A6TC15	Chorismate synthase	<0.05	<0.001
A6T8V1	Dipeptidyl carboxypeptidase	<0.05	<0.001
A6T5I3	DNA-binding protein HU-beta	<0.05	<0.001
A6TFP8	DNA-directed RNA polymerase subunit omega	<0.05	<0.001
E2QPN9	GltX	<0.05	<0.001
A6T4S1	Glucose dehydrogenase	<0.05	<0.001
A6T7S2	Glutamate dehydrogenase	<0.05	<0.001
E2QF55	Hypoxanthine phosphoribosyltransferase	<0.05	<0.001
A6TI00	Lipoate-protein ligase A	<0.05	<0.001
A6TAH5	Lipoprotein	<0.05	<0.001
A0A061YB63	Lysine--tRNA ligase	<0.05	<0.001
C9E714	OmpF	<0.05	<0.001
A6T631	Outer membrane porin, receptor for ferric enterobactin and colicins B and D fepA	<0.05	<0.001
A6THD2	Peptide methionine sulfoxide reductase	<0.05	<0.001
A6TGQ1	Phosphomethylpyrimidine synthase	<0.05	<0.001
A6TCB4	Phosphoribosylglycinamide formyltransferase	<0.05	<0.001
A6TG82	Protein FdhE homolog	<0.05	<0.001
A6TEB0	Putative enzyme, ferredoxin reductase-like	<0.05	<0.001
A6T5H7	Putative lipoprotein	<0.05	<0.001
A6T985	Putative LysM domain	<0.05	<0.001
A6TEG9	Putative periplasmic protein	<0.05	<0.001
A6T5Q0	Putative thioredoxin protein	<0.05	<0.001
A6TCF0	Putative uncharacterized protein	<0.05	<0.001
A6TB03	Putative uncharacterized protein	<0.05	<0.001
A6TE18	Putative uncharacterized protein	<0.05	<0.001
A6TEJ9	Putative uncharacterized protein	<0.05	<0.001

A6TEP1	Putative uncharacterized protein	<0.05	<0.001
A6TGL4	Putative uncharacterized protein	<0.05	<0.001
A6TGB9	Regulator of ribonuclease activity A	<0.05	<0.001
A6T7G8	Respiratory NADH dehydrogenase	<0.05	<0.001
A6T7J8	Response regulator in two-component regulatory system with PhoQ	<0.05	<0.001
A6TCL6	Ribosome maturation factor	<0.05	<0.001
A6TGP9	Thiamine biosynthesis protein	<0.05	<0.001
A6TGQ0	Thiamine-phosphate synthase	<0.05	<0.001
A6TFA0	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	<0.05	<0.001
A6T4W7	UPF0325 protein	<0.05	<0.001
A6TCJ2	Uracil-DNA glycosylase	<0.05	<0.001
A6T532	Xanthine phosphoribosyltransferase	<0.05	<0.001
A6TF02	Peptidyl-prolyl cis-trans isomerase	0.48	0.017
A6TFR2	Glycerol kinase	0.48	0.020
A6T693	D-alanyl-D-alanine carboxypeptidase, fraction A; penicillin-binding protein 5	0.47	0.012
A6TBN8	GTP cyclohydrolase	0.47	0.044
A6T5M4	AcrB	0.47	0.018
A6T4E9	Molybdenum cofactor biosynthesis protein	0.46	0.022
A6TG48	Aspartate--ammonia ligase	0.45	0.028
A6T4Y2	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	0.45	0.032
A6T5R1	Peptidyl-prolyl cis-trans isomerase	0.45	0.027
A6TBS4	Ecotin	0.45	0.025
A6T6C9	Flavodoxin	0.45	0.037
A6TGG1	Ketol-acid reductoisomerase	0.45	0.008
A6TF30	Putative uncharacterized protein	0.44	0.014
A6T5D9	Preprotein translocase subunit	0.44	0.015
A6TF37	Fe/S biogenesis protein	0.44	0.050
A6T4Q6	Transcriptional regulator of pyruvate dehydrogenase complex	0.42	0.030
A6THH2	Putative uncharacterized protein	0.40	0.028
A6T793	NAD(P)H dehydrogenase	0.39	0.005
A6TEL1	Putative transport protein	0.39	0.019
A6TCD7	Nucleoside diphosphate kinase	0.39	0.013
A6TBX0	NADH dehydrogenase	0.38	0.018
A6TFK6	Putative phosphatase	0.37	0.023
A6TB20	Keto-hydroxyglutarate-aldolase	0.37	0.001
A6TGH0	Thioredoxin	0.35	0.014
A6TF45	sn-glycerol-3-phosphate dehydrogenase	0.35	0.010
A6TER1	Suppresses inhibitory activity of CsrA	0.34	0.035
A6T7H0	Putative TonB-dependent receptor	0.34	0.004
A6TFG2	Putative outer membrane protein	0.34	0.004
A6TCT2	Putative uncharacterized protein	0.34	0.038
A6TGW7	Single-stranded DNA-binding protein	0.33	0.007
J7Q9Y1	ABC transporter maintaining OM lipid asymmetry	0.33	0.011
A6T7F4	Acyl carrier protein	0.33	0.005
A6TGQ7	DNA-binding protein	0.31	0.011

A6T6Y8	Outer-membrane lipoprotein carrier protein	0.30	0.008
A6TCJ1	Autonomous glycyl radical cofactor	0.30	0.006
A6T5E9	N utilization substance protein B homolog	0.29	0.001
A6TEH9	Putative enzyme	0.28	0.017
A6T6G7	Peptidoglycan-associated lipoprotein	0.28	0.009
A6T685	Cold shock protein	0.27	0.006
A6T4V4	Outer membrane pore protein, receptor for ferrichrome	0.26	0.001
A6T6Q8	OmpX	0.26	0.004
A6T9R8	Putative receptor	0.24	0.016
A6TAW4	D-amino acid dehydrogenase	0.21	0.045
A6TDK6	Putative fimbrial-like protein	0.11	0.003
A6TAL7	Nitrate reductase 1, delta subunit	<0.05	<0.001
A6TF14	Shikimate kinase 1	<0.05	<0.001
A6TCI1	Ribonuclease 3	<0.05	<0.001
A6TB01	Carboxy-terminal protease for penicillin-binding protein 3	0.41	0.022
A6T7Y9	Orotidine 5'-phosphate decarboxylase	<0.05	<0.001
L0BW80	Aminoglycoside 6'-N-acetyltransferase	<0.05	<0.001
A6TH52	10 kDa chaperonin	0.37	0.020

Table 31. Whole cell proteomics results of R13 1M compared to the derived mutant 1 Imi. Whole cell proteomics were prepared in triplicate. Fold change and t-test indicates the average of the three repeats. Accession numbers highlighted in green show proteins significantly (*p* value = <0.05) up-regulated, with a fold change of >2, in the mutant 1 Imi. Accession numbers highlighted in red show proteins significantly (*p*-value = <0.05) down-regulated with a fold change of <0.5.

4.6. Summary of phenotype and genotype of clinical isolates and selected mutants

Strain	Phenotype	Genotype
R13 CTX-M-15	RamR and OmpK35 loss	*ΔramR, ΔompK35*
1M (derived from R13)	RamR and OmpK35 loss, down-regulation of OmpK36 and LamB2	**ΔramR, ΔompK35*
1 Imi (derived from 1M)	RamR and OmpK35 loss, down-regulation of OmpK36 and LamB2	**ΔramR, ΔompK35*
8CAZ (derived from R13	RamR and OmpK35 loss, up-regulation of CTX-M	**ΔramR, ΔompK35*
R30 KPC-3	RamR, OqxR and OmpK35 loss	*ΔramR, ΔoqxR, ΔompK35*
2M (derived from R30)	RamR, OqxR and OmpK35 loss, down-regulation of OmpK36 and LamB2	**ΔramR, ΔoqxR, ΔompK35*
R21 CTX-M	RamR loss	*ΔramR*
2CAZ (derived from R21 CTX-M)	RamR loss, up-regulation of CTX-M	**ΔramR*
R21 OXA-232	RamR loss	*ΔramR*
16M (derived from R21 OXA)	RamR and OmpK36 loss	*ΔramR, ΔompK36*
R11 OXA-232 CTX-M-15	RamR and OmpK35 loss	*ΔramR, ΔompK35*
64M (derived from R11)	RamR, OmpK35 and OmpK36 loss	*ΔramR, ΔompK35, ΔompK36*
64M8CAZ (derived from 64M)	RamR, OmpK35 and OmpK36 loss, down-regulation of NlpD	**ΔramR, ΔompK35, ΔompK36*

Table 32. Summary of phenotypes and genotypes present in clinical isolates and selected mutants. * indicates missing genotype, either due to results not being returned or the inability to find a mutation (see chapter 5).

4.7. Combining meropenem/vaborbactam with avibactam/ceftazidime

Ceftazidime/avibactam is another β-lactam/β-lactamase inhibitor combination already licensed for clinical use. Since we could not find any β-lactam that could be universally efficacious against clinical isolates and mutants when used in combination with meropenem/vaborbactam, we decided to test the efficacy of combining ceftazidime/avibactam with meropenem/vaborbactam to combat β-lactam/vaborbactam resistance. All mutants made throughout this project were tested against ceftazidime/avibactam in combination with meropenem/vaborbactam using chequerboards. In fact, all mutants are susceptible to meropenem/vaborbactam in the presence of avibactam, even if there is no ceftazidime. Indeed, all the mutants are susceptible to ceftazidime/avibactam in the presence of vaborbactam, even if meropenem is not present. Hence, ceftazidime/avibactam plus meropenem/vaborbactam has great clinical potential against even these highly resistant mutants.

A) R13 1M Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

B) R13 1 Imi Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

C) R21 2CAZ Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

D) R13 8CAZ Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

E) 2M Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

A) 64M Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

F) 64M8CAZ Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

G) R21 OXA 16M Ceftazidime (Y axis) versus meropenem (X axis). Avibactam and vaborbactam are present in all wells.

	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
0												
0.5												
1												
2												
4												
8												
16												
32												

Figure 33. Chequerboard MICs of CTX-M, KPC and OXA β-lactam/vaborbactam mutants combining ceftazidime/avibactam and meropenem/vaborbactam. Cells filled in grey indicate bacterial growth, cells filled white indicate no bacterial growth. Concentration highlighted in red shows the non-susceptible cut-off (growth above these concentrations indicates non-susceptibility). Cell filled in green indicates the MIC of Ceftazidime/Avibactam in the presence of meropenem/vaborbactam. meropenem concentration is on the X-axis with ceftazidime concentration on the Y-axis. Vaborbactam and avibactam are present in each well at a final concentration of 8 µg/ml and 10 µg/ml respectively.

4.8. Conclusion

The first aim of this chapter was to select for in vitro produce *in vitro* meropenem/vaborbactam resistant mutants of clinical isolates, again either producing CTX-M, KPC or OXA-232 and to characterise the resistant mechanism. Here we find that the two OXA-232 *in vitro* mutants had mutations in *ompk36,* resulting in loss of Ompk36. Conversely, no ORF or promoter *ompk36* mutation could be found in the CTX-M or KPC meropenem/vaborbactam mutants ‘1M’ or ‘2M’, indicating an alternative resistance mechanism (see chapter 5).

The second aim of this chapter was to test a range of alternative vaborbactam partners that could extend activity to also include OXA-232 and also overcome meropenem/vaborbactam resistance in CTX-M and KPC producers. Here we find that ceftazidime shows potential as an alternative partner for vaborbactam against OXA-232 and CTX-M producers. We also find that imipenem shows potential as an alternative partner for vaborbactam against meropenem/vaborbactam resistant CTX-M producers. Conversely, we were unable to identify a β-lactam/vaborbactam combination capable of overcoming meropenem/vaborbactam resistance in the KPC producer 2M. Although we were unable to identify a universal β-lactam/vaborbactam capable of targeting OXA-232 and meropenem/vaborbactam resistant CTX-M and KPC producers, we present here the first use of combining two β-lactam/ β-lactamase inhibitor combinations. All isolates and in vitro mutants generated remained susceptible to ceftazidime/avibactam-meropenem/vaborbactam.

The final aim of this chapter was to identify and characterise mutants resistant to these alternative β-lactam-vaborbactam combinations. He we find that ceftazidime/vaborbactam resistance in CTX-M producers is due to over-expressing of the CTX-M enzyme. We believe this to increase the ceftazidime hydrolysis potential of the CTX-M enzyme even in the presence of the inhibitory effects of vaborbactam. Correspondingly, ceftazidime/vaborbactam resistance in OXA-232 producers, seems to be due to permeability and efflux changes. However, proteomics indicated no obvious discernible mechanism of resistance.

Chapter 5: Discussion and Future work

The first part of this study sought to better understand meropenem/vaborbactam resistance in *K. pneumoniae*. This was achieved both through the construction of a panel of isogenic, permeability and efflux modified strains as well as the *in vitro* of meropenem/vaborbactam resistant mutants. It was found that OmpK36 loss or down-regulation was the strongest indicator of meropenem/vaborbactam resistance in both the panel of otherwise isogenic Ecl8 derivatives and the meropenem/vaborbactam resistant mutants of clinical isolates. This seems to concur with the current literature as CRE has frequently been observed due to the carrying of carbapenemases in combination with porin loss and overproduction of efflux pumps. These carbapenem resistance indicators also seem to show a similar effect on meropenem/vaborbactam susceptibility. OmpK35 loss or downregulation also plays an important role in meropenem/vaborbactam non-susceptibility. Both meropenem and vaborbactam can enter through OmpK36 and OmpK35, although vaborbactam seems to show a greater preference for OmpK36 mediated entry (165). This explains why OmpK36 shows a greater effect when compared to OmpK35. It has been hypothesised that loss of one of the major porins, either OmpK36 or OmpK35 could lead to competition for passage into the cell, increasing the importance of the other.

Upon analysis of the whole genome sequence of the four meropenem/vaborbactam selected mutants. Two mutants showed mutational inactivation of *ompK36*. The OXA-232 mutant 64M contained a 263bp insertion sequence in the upstream promoter region of the *ompK36* gene, which was not present in the parental clinical isolate R11. The OXA-232 mutant 16M showed *ompK36* truncation via the introduction of a stop codon. The other two mutants showed an unchanged *ompK36* open reading frame and promoter sequence. Additionally, no mutation could be found in the open reading frame or promoter sequence on the two-component system *ompR/envZ*, which is known to play a role in *ompC* and *ompF* regulation in *E. coli* (247). This could indicate that merely down-regulating the expression of OmpK36 is sufficient to cause meropenem/vaborbactam non-susceptibility, and that there are currently unknown regulators in *K. pneumoniae* that control OmpK36 production. Alternatively, several other outer membrane proteins have previously been implicated in carbapenem resistance in *K. pneumoniae*. These are: OmpK26, the inducible OmpK37, the Maltoporin LamB and the phosphate-regulated porin PhoE (199, 248-250). OmpK26 and OmpK37 seem to be compensatory porins to maintain viability and allow import of nutrients as both OmpK26 and OmpK37 are significantly repressed under normal cell conditions but are often upregulated in strains that show loss of either OmpK35 or OmpK36 (248, 251). Although one meropenem/vaborbactam resistant mutant did show a significant change in abundance of OmpK26, this was a down-regulation event, with a fold-change of 0.22. Therefore, we do not believe that OmpK26 is playing a role in meropenem/vaborbactam susceptibility in any of

these mutants. Loss or down-regulation of PhoE and LamB have previously been reported as having a role in carbapenem resistance in *K. pneumoniae*, however, their role in meropenem/vaborbactam resistance has not been described (199, 249). No observable change in abundance of PhoE was seen in any of the meropenem/vaborbactam resistant mutants, however down-regulation of LamB was found in both the CTX-M-15 mutant 1M and the KPC-3 mutant 2M with fold changes of 0.31 and 0.29 respectively alongside OmpK36 downregulation. Again, no mutation could be found in the *lamB* open reading frame or promoter region. Perhaps the (potentially novel) mechanism of OmpK36 control also controls LamB porin production.

Although there is limited knowledge regarding *lamB* in *K. pneumoniae,* in *E. coli lamB* is known to be located in the *malKlamBmalM* operon which combines with another operon *malEFG* (252). It is thought that that the promoter regions of these operons could overlap and that both operons are controlled by the positive regulatory gene *malT* (253). Furthermore in *E. coli* and *Salmonella*, the expression of LamB is known to be influenced through the expression of the inhibitory antisense microRNA *micA*, in a similar mechanism as seen with *micF* repression of *ompK35* mRNA translation (219, 254). The levels of *micA* have been shown to be controlled by both the stress alarmone ppGpp and sigma factor E (255). It is unclear whether mutations in either ppGpp or sigma factor E could lead to down-regulation of LamB. Therefore, the control of LamB is an incredibly complex signalling network combining positive and negative gene regulation with post transcriptional repression through the action of *micA*. The role of LamB in meropenem/vaborbactam susceptibility requires significant further investigation. Future work could involve the inclusion of long read whole genome sequencing to 'finish' the genomes of both the clinical isolates and the generated mutants allowing direct mapping to one another. The short comings of short read whole genome sequence is that if there is not a suitable, fully annotated, reference genome to map to, identification of SNPs is problematic. We found that the clinical isolates used throughout this project showed high variability when mapped to the reference genome GCF_000315385.1 making the identification of SNPs difficult. Therefore, it is highly probable that genuine mutations in candidate genes such as *ompK36*, *lamB*, *phoE* and their regulators could have been missed.

The second part of this project sought to find if any other β-lactams could potentiate the activity of meropenem/vaborbactam. This was attempted because it is possible for clinicians to administer meropenem/vaborbactam with a β-lactam at the same time to the same patient, and so this work was designed to inform potential combination therapy when meropenem/vaborbactam emerges – as it surely will – in the clinic. Given that we have predicted this will happen in strains with multiple permeability defects: loss of ompK35, ramR

and then downregulation of OmpK36, we were therefore specifically whether β-lactams in combination with meropenem/vaborbactam could overcome meropenem/vaborbactam resistance (caused by OmpK36 downregulation/loss) in a CTX-M-15 producer with a *ramR* and *ompK35* mutation (R13), a KPC-3 producer with *ramR, oqxR* and *ompK35* mutations (R30) and finally an OXA-232, CTX-M-15 producer with *ramR* and *ompK35* mutations (R11). Imipenem was shown to rescue meropenem/vaborbactam resistance in the meropenem/vaborbactam resistant mutant, CTX-M-15 producer, 1M, though further selection of imipenem/vaborbactam resistance was straightforward. Upon trying to characterise imipenem/vaborbactam resistance using LC-MS/MS proteomics, no obvious mechanism of resistance could be discerned. Once the whole genome sequencing results have been returned it may become clearer what the mechanism of resistance is, but we do believe it do be novel. Nonetheless, this shows a clear limitation for imipenem/meropenem/vaborbactam combination therapy.

Ceftazidime/meropenem/vaborbactam was shown to be a combination that worked against the meropenem resistant mutants 1M and 64M (derived from the OXA-232, CTX-M-15 producing R11) but again resistance to this combination was easily lost by mutation. Characterising ceftazidime/meropenem/vaborbactam resistance showed up-regulation of CTX-M-15 as the mechanism of resistance in two such mutants. We believe this to increase the ceftazidime hydrolysis potential of the CTX-M enzyme even in the presence of the inhibitory effects of vaborbactam. However, ceftazidime/vaborbactam resistance in the OXA-232, CTX-M15 ceftazidime/vaborbactam resistant mutant 64M8CAZ (derived from 64M), showed no such up-regulation of CTX-M. Instead, a novel cephalosporin resistance mechanism was indicated which apparently involves down-regulation of the lipoprotein NlpD. In *E. coli, nlpD* has been characterised as forming an operon with *rpoS* and NlpD plays a role in the modelling and maintenance of the outer membrane (256, 257). It also plays an important role in peptidoglycan remodelling, by the activation of a peptidoglycan hydrolase AmiC, to carry out cytokinesis (258, 259). It has been reported that mutants lacking *nlpD,* exhibit a phenotype comparable to one which has outer membrane defects (258). Interestingly, PhD student Wan Nur Ismah selected cefuroxime resistant mutants of Ecl8 derivative 4-4 and identified downregulation of NlpD. It seems therefore, that this is a novel cephalosporin resistance mechanism. However, in *K. pneumoniae*, the role of *nlpD* remains to be characterised. To better understand whether NlpD is indeed an indicator of ceftazidime/vaborbactam resistance, a knockout mutant of *nlpD* in R11 could be made to test whether this gives the same phenotype and proteomics profile as the ceftazidime/vaborbactam resistant mutant.

Unfortunately, therefore, no β-lactam/meropenem/vaborbactam combination could be identified which was universally efficacious against the clinical isolates and mutants generated throughout this project. However, perhaps the most clinically important finding of this work, is that utilisation of ceftazidime/avibactam in combination with meropenem/vaborbactam showed great promise and rescued meropenem/vaborbactam resistance in all clinical isolates and mutants tested. A recent report identified the potential for using both ceftazidime/avibactam and meropenem/vaborbactam as a more favourable therapeutic option over colistin-based regimens (260). The work presented in this project is the first reported use of meropenem/vaborbactam in combination with ceftazidime/avibactam *in vitro* against characterised clinical isolates and resistant mutants. We have seen that ceftazidime/avibactam rescues meropenem/vaborbactam resistance, however, further work is required to evaluate whether meropenem/vaborbactam can restore ceftazidime/avibactam susceptibility in mutants having mutations in the avibactam target enzymes KPC and CTX-M. To test this, it would be necessary to introduce point mutations into *blaKPC* (D179Y/T243M double substitution, D179Y or V240G) and *blaCTX-M (*P170S/T264I double substitution) that confer ceftazidime/avibactam resistance and then carry out antimicrobial susceptibility testing of ceftazidime/avibactam and ceftazidime/avibactam in combination with meropenem/vaborbactam (168, 169). Meropenem has previously been used to treat infections caused by ceftazidime/avibactam resistant isolates (due to the presence of a D179Y/T243M double substitution in the KPC-3 enzyme, which actually makes it a worse carbapenemase), (261). Therefore, we have provided significant evidence that combining meropenem/vaborbactam with ceftazidime/avibactam will restore susceptibility against isolates expressing meropenem/vaborbactam resistance or ceftazidime/avibactam resistance alone, and this combination could act as “salvage therapy” for an increasing number of clinical cases in years to come.

Chapter 6: Bibliography

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